Photo-fermentative treatment of wastewaters: surveying local sources and examining their treatment by *Rhodopseudomonas palustris*

by

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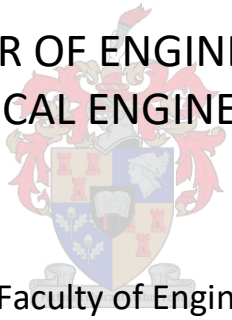

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ABSTRACT

Owing to rapid industrialization, environmental pollution in the form of industrial wastewater is a growing concern. Additionally, global energy requirements are mostly dependent on fossil fuels and at current fossil fuel consumption rates, these limited resources will inevitably be depleted. In order to counteract the exhaustion of fossil fuels as well as to compensate for their negative impact on the environment, hydrogen has been proposed as a future energy provider. The experimental work completed throughout this research arose due to the need for high organic content industrial wastewater treatment. South African industries are both major users and polluters of water, and produce wastewater streams which are often rich in recalcitrant organic compounds. This project aims to develop a biotechnology process which is able to both reduce the organic loading in these wastewaters and produce a valuable commodity product, in the form of hydrogen gas. This is achieved through an anaerobic photo-fermentation process whereby the bacterium *Rhodopseudomonas palustris* is able to consume the organic portion of the wastewater. The main aims of this research are firstly to conduct a survey of local literature and industries to determine which organic containing waste streams have the potential to be treated through photo-fermentation, secondly to evaluate the ability of *R. palustris* to metabolize a variety of waste organic components by generating growth curves when various wastewaters are used as substrates and lastly, to evaluate the wastewater treatment potential and hydrogen production potential of *R. palustris*. Wastewater from several South African industries was obtained. These include winery, anaerobic digester (AD) effluent, brewery, vinasse waste, olive processing, tannery, fish processing, paper and pulp and textile dye wastewater. For each effluent, a batch photo-fermentative growth and wastewater treatment experiment was conducted with free cells at various dilution rates with carbon and nitrogen free media. Significant growth of *Rhodopseudomonas palustris* was observed on these wastewaters accompanied by a reduction in chemical oxygen demand (COD). Undiluted winery wastewater proved to be most suitable for photo-fermentative growth and wastewater treatment, exhibiting a 74 % increase in biomass and a 72 % reduction in COD over a 21 day period. Wastewater treatment experiments were then conducted on the undiluted winery wastewater with cells immobilized in polyvinyl alcohol (PVA) cryogels and under continuous operation so as to simulate industrial treatment processes. A consistent COD reduction of approximately 5 % was achieved over a 28 day period when the bioreactor was operated continuously. It was concluded that the overall COD reduction was lengthy (21 days) and commonly longer than current alternative wastewater treatment technologies. However, this was a scoping study which focussed on the photo-fermentative treatment of organic containing industrial wastewaters without any pre-treatment steps. With adequate pre-treatment steps this bio process can be modified to produce hydrogen gas. Therefore, in spite of the lengthy COD reduction, *R. palustris* remains a

candidate organism for wastewater treatment owing to the possible production of valuable commodity products.

ABSTRAK

Weens spoedige industrialisasie is omgewingsbesoedeling in die vorm van industriële afvalwater 'n groeiende kommer. Boonop is globale energie vereistes meestal afhanklik van fossielbrandstowwe en teen huidige tempo's van fossielbrandstofgebruik, sal hierdie beperkte hulpbronne onvermydelik uitgeput word. Om die uitputting van fossielbrandstowwe teen te werk, sowel as om te kompenseer vir hul negatiewe impak op die omgewing, is waterstof voorgestel as 'n toekomstige energie verskaffer. Die eksperimentele werk voltooi deur hierdie navorsing het ontstaan as gevolg van die behoefte vir hoë organiese inhoud industriële afvalwaterbehandeling. Suid-Afrikaanse industrieë is beide groot gebruikers en besoedelaars van water en vervaardig waterafvalstrome wat gereeld ryk is aan weerspannige organiese samestellings. Hierdie projek beoog om 'n biotegnologie te ontwikkel wat beide die organiese lading in hierdie afvalwater kan verminder en 'n waardevolle kommoditeitsproduk kan vervaardig, in die vorm van waterstofgas, deur 'n anaerobierse fotoferementasieproses waar die bakterie *Rhodopseudomonas palustris* die organiese deel van die afvalwater kan verteer. Die hoofdoelstellings van hierdie navorsing is om i) 'n opname te maak van plaaslike literatuur en industrieë om te bepaal watter organies-houdende afvalstrome die potensiaal het om behandel te word deur fotofermentasie, ii) om die vermoë van *R. palustris* om 'n verskeidenheid afval organiese komponente te metaboliseer, te evalueer deur groeikurwes te genereer as verskeie afvalwaters as substrate gebruik word, en laastens iii) om die afvalwaterbehandeling potensiaal en waterstofproduksie potensiaal van *R. palustris* te evalueer. Afvalwater van verskeie Suid-Afrikaanse industrieë is verkry. Hierdie sluit in wynmakery, AD rioolwater, brouery, vinasse afval, olyfprosessering, leerlooiery, visprosessering, papier-en-pulp- en kleurstofafvalwater. Vir elke afloopwater is 'n lot fotofermentasie groei en afvalwaterbehandelingseksperiment uitgevoer met vrye selle en teen verskeie verdunningstempo's met koolstofvrye- en stikstofvrye-media. Beduidende groei is waargeneem op hierdie afvalwater wat gepaard gegaan het met chemiese suurstofvraag (COD) verwydering. Onverdunde wynmakery-afvalwater is bewys as die mees gepaste afvalwater vir fotofermentasie groei en afvalwaterbehandeling, met 'n 74% verhoging in biomassa en 'n 72% afname in COD oor 'n 21 dae periode. Afvalwaterbehandelingeksperimente is toe uitgevoer op die onverdunde wynmakery-afvalwater met selle geïmmobiliseer in poliviniel alkohol (PVA) kriojel en kontinue bedryf sodat industriële behandelingsprosesse gesimuleer word. 'n Konsekwente COD-reduksie van ongeveer 5% is bereik oor 'n 28 dae periode toe die bioreaktor kontinu bedryf is. Dis bepaal dat die algehele COD-reduksie langdurig en oor die algemeen langer as huidige alternatiewe afvalwaterbehandelingtegnologieë is. Hierdie was egter 'n bestekstudie wat gefokus het op die fotofermentasiebehandeling van organies-houdende industriële afvalwater sonder enige voorbehandelingstappe. Met voldoende voorbehandelingstappe kan hierdie bioproses gewysig word om waterstofgas te vervaardig. Daarom, ten spyte van die langdurige COD-reduksie, bly *R. palustris* 'n

kandidaat organisme vir afvalwaterbehandeling vanweë die moontlike vervaardiging van waardevolle kommoditeitsprodukte.

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NOMENCLATURE

Description	Symbol	Unit
Nicotinamide adenine dinucleotide (reduced)	NADH	-
Nicotinamide adenine dinucleotide (oxidized)	NAD^+	-
Adenosine triphosphate	ATP	-
Adenosine diphosphate	ADP	-
Electron	e^-	-
Phosphate	P_i	-
Generalised organic compound	$C_XH_YO_Z$	-
Wastewater	WW	-
Chemical oxygen demand	COD	-
Suspended solids	SS	-
Total dissolved solids	TDS	-
Total organic carbon	TOC	-
Adsorbable organic halides	AOX	-
Specific effluent volume	SEV	-
Fats, oils and grease	FOG	-
Soaps, oils and grease	SOG	-
Total phosphorus	TP	-
Initial absorbance	A_i	-
Final absorbance	A_f	-
Optical density	OD	-

1. INTRODUCTION

Today global energy requirements are mostly dependent on fossil fuels and at current fossil fuel consumption rates, these limited resources will inevitably be depleted. Presently, the utilization of fossil fuels are causing global climate change mainly due to the emission of pollutants which are released into the atmosphere as a result of combustion processes. In order to counteract the exhaustion of fossil fuels as well as to compensate for their negative impact on the environment, hydrogen has been proposed as a future energy provider. Despite not being a primary source of energy, hydrogen aids in the storage and utilization of primary energy sources, and is considered to be a clean and non-polluting fuel which is harmless to both humans and animals (Crabtree, Dresselhaus and Buchanan, 2004) (Ipcc, 2007).

Owing to rapid industrialization and urbanization, environmental pollution in the form of industrial wastewater is a great and growing concern. This increase in environmental pollution is linked to a concern for development of alternative water treatment bio-technologies. Current water treatment technologies are expensive, energy intensive and not much benefit is derived from the wastewater. Furthermore, using current methods, these wastewater streams have also proven difficult to treat within legislative discharge standards. With industrial wastewaters containing large amounts of biodegradable organic compounds which are ideal substrates for microbial growth, much benefit can still be derived from the treatment of these wastewaters (Das, 2001).

Photosynthetic bacteria produce hydrogen from, for example, organic acids in a light dependent reaction known as photo-fermentation. Hydrogen is generated from substrates through the input of additional energy via bacterial photosynthesis. Previous research (Keskin, Abo-Hashesh and Hallenbeck, 2011) has focussed on the conversion of organic acids into hydrogen using this photo-fermentative process. These organic acids have occasionally been sources as substrates from waste streams, however the majority of the work done has been on pure substrate conversions. There is an increasing focus therefore on the expansion of research (Keskin, Abo-Hashesh and Hallenbeck, 2011) on substrate utilisation. This includes examining other organic compounds found in wastewaters which have been identified as effective in a photo-fermentative process. The characteristics of this process allow for virtually stoichiometric conversions of the various substrates to hydrogen. However, many noteworthy problems and obstacles remain. These include low light conversion efficiencies as well as the high energy demand and low turnover number of nitrogenase. Further, expensive photo-bioreactors are potentially required (Hallenbeck and Liu, 2016).

This project aims to develop a biotechnology process which is able to both treat and purify wastewater, in terms of reducing pollutant concentrations, as well as produce a clean, high energy fuel in the form

of hydrogen gas. This is achieved through an anaerobic photo-fermentation process whereby the bacterium *Rhodopseudomonas palustris* is able to consume the organic portion of the wastewater and produce both hydrogen gas and a partially purified water stream.

2. AIMS AND OBJECTIVES

1. Conduct a survey of literature and local industries to determine which organic containing waste streams require treatment.
2. Determine which of these waste streams has the potential to be treated through photo-fermentative hydrogen production.
3. Evaluate the ability of *R. palustris* to metabolise a variety of waste organic components, including anaerobic digestion effluent, phenolic industrial effluent and various other recalcitrant compounds.
4. Evaluate the industrial wastewater treatment potential of *R. palustris*.
5. Evaluate the ability of *R. palustris* to produce hydrogen from industrial wastewater.

If successful, the project will develop a viable wastewater treatment process which has the potential to impact both industrial and municipal levels through its application to recalcitrant organic wastes and the concomitant generation of hydrogen.

3. LITERATURE REVIEW

3.1. Rhodopseudomonas palustris

3.1.1. Background

R. palustris is a purple non-sulphur bacterium which is rod shaped and gram negative. Further, it is renowned for its diverse metabolism and is able to switch between the four modes of metabolism which support life. This indicates that it can survive photoautotrophically, photoheterotrophically, chemoautotrophically and chemoheterotrophically. These modes of growth are summarized in Table 1 (Pott, 2013).

Table 1: R. palustris modes of growth.

Mode of growth	Oxygen requirement	Light requirement	Source of carbon	Energy source (electron source)	H_2 production
Chemoheterotrophic	Yes	No	Organic carbon	Organic carbon	No
Chemoautotrophic	Yes	No	CO_2	H_2, thiosulphate and other inorganic electron donors	H_2 consumption
Photoautotrophic	No	Yes	CO_2	H_2, thiosulphate and other inorganic electron donors/light	H_2 consumption
Photoheterotrophic	No	Yes	Organic carbon	Organic carbon/light	Yes (in the absence of ammonia)

Photosynthetic purple non-sulphur bacteria such as *R. palustris* are extensively dispersed in the natural environment. Specifically, these organisms are particularly found in organically polluted water sources. In nature, they perform vital roles in terms of purifying and cleaning water sources and the environment as they are able to consume many organic pollutants in a phototrophic process. The metabolic advantages of *R. palustris* are that they can grow in wastewaters with high organic loading and are able to reduce the chemical oxygen demand (COD) of the wastewater. Additionally, this process can also be modified to produce hydrogen gas and bacterial biomass which can be used as an agricultural feedstock in the poultry and aquaculture industries. This also means that they are potential

candidate organisms for applications in industrial wastewater treatment (Getha, Vikineswary and Chong, 1998).

Purple photosynthetic bacteria including *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodovulum sulfidophilum* and *Rhodopseudomonas palustris* have been reported as having the ability to produce hydrogen gas (Kapdan and Kargi, 2006). This is useful as an additional benefit from a process engineering perspective in terms of energy recovery, as well as the production of a value added commodity product.

3.1.2. Modes of metabolism

Microbial metabolism refers to the methods by which bacteria attain the nutrients, for instance carbon, and energy necessary to reproduce and live. Microbial metabolisms are arranged according to three categories: how the organism obtains carbon for synthesizing cell mass, how the organism obtains reducing equivalents used for either energy conservation or for biosynthetic reactions and how the organism obtains energy for living and growth. *R. palustris* can grow both aerobically or anaerobically and can utilize organic and inorganic compounds as well as light as energy sources. It is also able to fix nitrogen for growth. Finally, *R. palustris* can also obtain carbon from either organic compounds or from the fixation of carbon dioxide. Research has been conducted as to how *R. palustris* adjusts its metabolism in response to environmental variations and stimuli. The organism is able to obtain and process different environmental components such as variations in light, oxygen, carbon and nitrogen levels (Pott, 2013).

3.1.3 Fermentation

Fermentation is a particular kind of heterotrophic metabolism which makes use of organic carbon instead of oxygen as a terminal electron acceptor. This implies that these bacteria do not use an electron transport chain to oxidise NADH to NAD^+. This means that these organisms must have an alternative process by which to harness this reducing power and to maintain a supply of NAD^+ for the satisfactory functioning of normal metabolic pathways (Pott, 2013).

Fermentative organisms are anaerobic. Many facultative anaerobes, including *R. palustris*, support fermentation under anaerobic conditions or aerobic respiration. As a replacement for using ATP synthase as in respiration, ATP in fermentative organisms is either produced by substrate-level phosphorylation or by coupling the oxidation of low-energy organic compounds directly to the formation of a proton motive force. However, these reactions are extremely low-energy yielding (Pott, 2013).

During substrate-level phosphorylation a phosphate group is transferred from a high-energy organic compound to ADP to form ATP. As a result of the need to produce high energy phosphate-containing

organic compounds (generally in the form of Coenzyme A-esters) fermentative organisms use NADH and other cofactors to produce many different reduced metabolic by-products, often including hydrogen gas (H_2) (Pott, 2013).

3.1.4. Phototrophy

R. palustris is able to use light as an energy source to produce ATP and organic compounds such as carbohydrates, lipids and proteins. This happens in an anoxygenic photosynthetic process. As there is a wide variety of photosynthetic bacteria, many mechanisms exist which convert light into energy for metabolism. Photosynthetic reaction centres are located within a membrane by all photosynthetic bacteria. The flow of electrons is cyclic in anoxygenic photosynthetic bacteria. This means that all of the electrons used during photosynthesis are ultimately transferred back to the single reaction centre. In purple bacteria, the reduced chemical potential of this reaction centre results in the formation of NADH via reverse electron flow. However, in all circumstances, the generation of a proton motive is used to drive ATP production by means of an ATP synthase. The majority of photosynthetic microorganisms are autotrophic, achieving carbon dioxide fixation by means of the Calvin cycle. Certain photosynthetic bacteria, for instance *R. palustris*, are photoheterotrophs, indicating that they utilize organic carbon substances both as a source of carbon as well as a source of energy for growth. Additionally, certain photosynthetic bacteria are also capable of fixing nitrogen (Pott, 2013).

3.1.5. Nitrogen fixation

All biological systems require nitrogen as an essential element for growth. Despite being abundant in the atmosphere, nitrogen gas (N_2) is usually biologically inaccessible owing to the large energy required to overcome the triple bond. In the natural environment, only particular bacteria are capable of nitrogen fixation. The process converts nitrogen gas into ammonia (NH_3) which can readily be assimilated by all organisms. These organisms are therefore extremely important from an ecological standpoint and are often vital for the survival of entire ecosystems. The enzyme responsible for nitrogen fixation is nitrogenase. This enzyme is extremely sensitive to oxygen which will inhibit it irreversibly. Nitrogen fixation is also an enormously energetically expensive process with 16 – 24 ATP are used per molecule of N_2 fixed. For these reasons, the production and activity of nitrogenase are greatly regulated and controlled (Pott, 2013).

The activity of nitrogenase is such that hydrogen is concomitantly produced during this process. Depending on which metal cofactor is present, ammonia and concomitant hydrogen will be produced according to equations 1, 2 and 3.

Mo: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$ [1]

Fe: $N_2 + 21H^+ + 21e^- + 42ATP \rightarrow 2NH_3 + 7.5H_2 + 42ADP + 42P_i$ [2]

V: $N_2 + 12H^+ + 8e^- + 24ATP \rightarrow 2NH_3 + 3H_2 + 24ADP + 16P_i$ [3]

Analysing equation 1, it is seen that 1 mole of hydrogen is produced per 16 moles of ATP consumed. This is an extremely energy intensive reaction. However, in the absence of molecular nitrogen to fix, the enzyme still operates to produce hydrogen, except in that case all the ATP energy is diverted to hydrogen production. In other words, when there is no nitrogen available for fixation, protons are reduced instead of nitrogen according to equation 4.

$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4P_i$ [4]

Analysing equation 4, it is seen that 1 mole of hydrogen is produced per 4 moles of ATP consumed. This is not an energy intensive reaction and is a vast improvement on reaction shown by equation 1. This reduction can be seen as the complete oxidation of the carbon substrate to hydrogen which is shown by equation 5.

$C_XH_YO_Z + (2X - Z)H_2O \rightarrow \left(\frac{Y}{2} + 2X - Z\right)H_2 + XCO_2$ [5]

ATP produced by photosynthesis is supplied to these reactions in order to overcome the energy deficit. It can be concluded that maximum hydrogen production will occur under nitrogen deficient conditions.

3.1.6. Metabolism involved in hydrogen production

See Figure 35 in Appendix C for a detailed insight into the metabolism involved in hydrogen production.

3.2. Waste streams

South Africa has significant resources in the form of waste streams, for example industrial or agricultural effluents. These effluents require treatment before they can be safely disposed of. *R. palustris* can be used to simultaneously treat these effluents while producing hydrogen as a commodity chemical, as well as other valuable by-products. The first aim of this project is therefore to identify these waste streams, and investigate the ability of *R. palustris* to photo-ferment the waste stream components (DEA, 2015).

3.2.1. Energy from waste

The Department of Energy aims to bring about integration of renewable energies into the mainstream energy economy. At the time, 2003, the national goal was set at 10 000 GWh renewable energy contribution to final energy consumption by 2013. The Department of Energy proposed that these

renewable energies would largely be generated from wind, solar, biomass and small hydropower plants. Further, the Department of Energy went on to propose that these renewable energies be used for non-electric technologies such as bio-fuels and solar water heating. At present, South Africa's primary and secondary energy requirements have continued to be mainly fossil fuel based. The use of fossil fuels is causing global climate change which is mainly due to the emission of pollutants released into the atmosphere as a result of the combustion process (DEA, 2015).

A promising renewable energy option is residual biomass and biofuels. Renewable sources of organic material are usually used to derive biomass energy. This energy can then be used to provide heat, manufacture liquid bio-fuels as well as for the generation of electricity. Common biomass categories include plants, agricultural residues, forestry residues and organic compounds in industrial and municipal waste streams. Bio-fuels are generated by the conversion of biomass and can be used as a substitute for fossil fuels. Characteristic applications of bio-fuels include transportation and internal combustion engines (DEA, 2015).

A special case of bio-fuels, bio-gas, generally refers to the gas which is generated through the biological breakdown of organic matter. Examples of organic containing waste streams include municipal wastewater and animal manure which can be converted to biogas via photo-fermentative and anaerobic digestion systems. Biogas can then be utilized as a fuel for cooking and heating, as well as for the production of electricity by means of biogas generators.
In industrialised countries, biogas plants are largely utilized for the generation of power via the conversion of biogas to electricity. This has become a typical and growing technology option in these countries (DEA, 2015).

3.2.2. Waste definitions

The following definitions of waste, general waste, hazardous waste and building and demolition waste are given in Table 2. These definitions of waste give an indication of the waste produced in South Africa and how the waste is classified.

Table 2: D definitions of waste, general waste, hazardous waste and building and demolition waste. Table *adapted from* ('NATIONAL WASTE INFORMATION BASELINE REPORT', 2012).

Waste	"Any substance, whether or not that substance can be reduced, re-used, recycled and recovered (a) That is surplus, unwanted, rejected, discarded, abandoned or disposed of; (b) Which the generator has no further use of for the purposes of production; (c) That must be treated or disposed of; or (d) That is identified as a waste by the Minister by notice in the Gazette, and includes waste generated by the mining, medical or other sector; but – (i) A by-product is not considered waste; and (ii) Any portion of waste, once re-used, recycled and recovered, ceases to be waste."
General waste	"Waste that does not pose an immediate hazard or threat to health or the environment, and includes: (a) Domestic waste; (b) Building and demolition waste; (c) Business waste; and (d) Inert waste."
Hazardous waste	"Any waste that contains organic or inorganic elements or compounds that may, owing to the inherent physical, chemical or toxicological characteristics of that waste, have a detrimental impact on health and the environment."
Building and demolition waste	"Waste, excluding hazardous waste, produced during the construction, alteration, repair or demolition of any structure, and includes rubble, earth, rock and wood displaced during that construction, alteration, repair or demolition."

In terms of municipal waste, only a limited number of waste characterisation studies have been completed in South Africa. Organic waste can be classified and categorized as either putrescible waste, garden waste or as greens. The summation of these organic waste categories leads to the overall picture of municipal organic waste. The municipal waste composition by mass is given in Figure 1 where the data for Gauteng and Cape Town is comparable ('NATIONAL WASTE INFORMATION BASELINE REPORT', 2012).

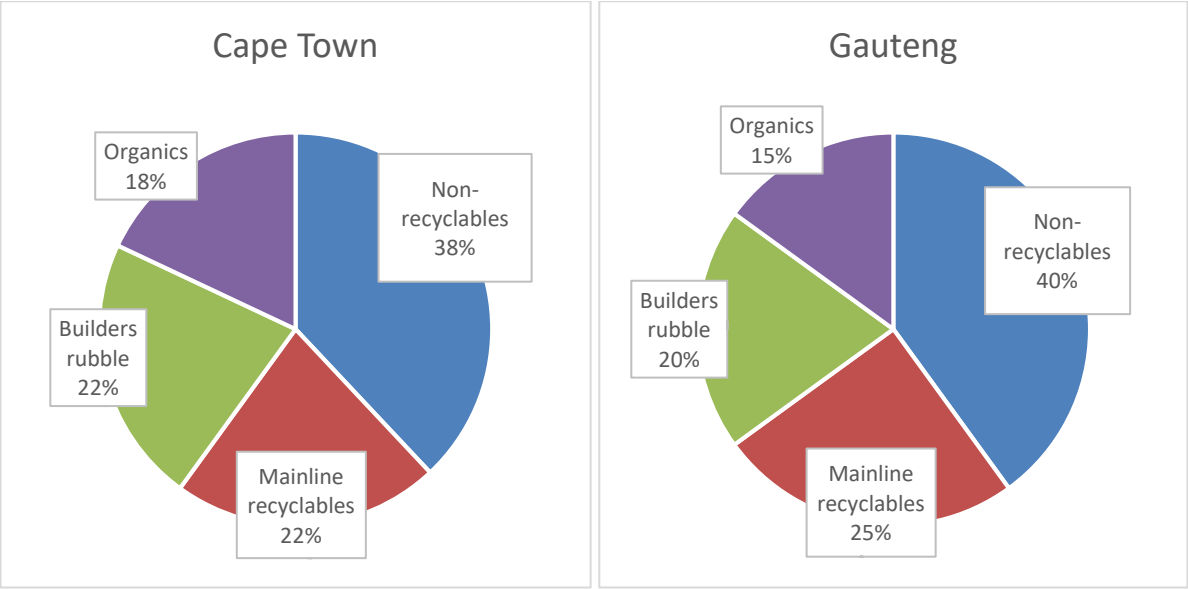

Figure *1: Municipal waste composition by mass.* Figure *adapted from* ('NATIONAL WASTE INFORMATION BASELINE REPORT', 2012).

Since there are no statististically significant differences between the municipal waste composition for Gauteng and Cape Town, the data can be extrapolated in order to provide a fair representation of municipal waste composition in South Africa. This leads to the assumption that municipal waste composition will be similar throughout South Africa ('NATIONAL WASTE INFORMATION BASELINE REPORT', 2012).

The waste category, mainline recyclables, includes paper, plastics, glass, tins and tyres. These waste streams are already reported under organic waste, construction and demolition waste, paper, plastic, glass, metals and tyres. The reason for this being that these waste streams include the recyclables from municipal waste as well as the recyclables collected directly from industrial sources. Nonetheless, a significant portion of waste is categorised as containing organics and can therefore be used as a material from which to derive benefit in the form of valuable products such as hydrogen ('NATIONAL WASTE INFORMATION BASELINE REPORT', 2012).

Through the modelling of relevant data, roughly 108 million tonnes of waste was generated in South Africa in 2011. Of this generated waste, 97 million tonnes were disposed of at landfill sites. Further, approximately 59 million tonnes is considered to be general waste and 49 million tonnes is considered to be unclassified and hazardous waste. Additionally, approximately 10 % of the total waste generated in South Africa is recycled annually. This allows for opportunities to increase waste recycle and valorisation in South Africa. For the case of organic waste, this can be achieved through biological treatment technologies instead of conventional waste management strategies such as land filling ('NATIONAL WASTE INFORMATION BASELINE REPORT', 2012).

An accurate determination of South Africa's waste information baseline has proven to be extremely challenging. This is mainly as a result of inaccurate waste data reporting. Not all waste streams are classified and certain waste streams have the possibility of being classified as either general or hazardous waste due to the difficulty in splitting the hazardous and non-hazardous waste components in the absence of analytical data. Nevertheless, the reported waste compositions are accepted as being satisfactorily accurate for the purposes of policy decisions ('NATIONAL WASTE INFORMATION BASELINE REPORT', 2012).

3.2.3. Wastewater characteristics

3.2.3.1. Water quality and threats to water quality

Water quality is threatened by many factors, most prominently from chemical pollution. Chemical pollution from heavy metals, solvents, dyes and pesticides enter aquatic environments in many ways. For instance, industrial wastewaters are often dumped directly into water sources or effluents are released from wastewater treatment plants which do not adhere to discharge requirements. Additionally, pollutants enter aquatic systems by means of agricultural pesticides and fertilizers. Discharge resulting from poor enforcement of laws and illegal practices also adds to water contamination (Oller, Malato and Sánchez-Pérez, 2011).

Previously, much focus was placed on identification of extreme individual polluting components. However, current focus is placed on remediation strategies aimed at chronic environmental issues related to the continual emission of pollutants. Additionally, greater amounts of recalcitrant compounds are being found at long distances from their discharge sources. The major avenues for decreasing toxic components in wastewater are photo-degradation and biodegradation. Photo-degradation has proven successful for the treatment of aromatic hydrocarbons, chlorinated aromatic hydrocarbons, chlorinated phenols, and many pesticides. Biodegradation refers to the process where chemicals are broken down by bacteria and fungi which are naturally found in aquatic and terrestrial environments. Special consideration has to be given to the type of microorganism used as well as to the source of the industrial wastewater. Many organic compounds produced by industry are toxic or resilient to biological treatment, thereby requiring pre-treatment steps or specific experimental operating conditions (Oller, Malato and Sánchez-Pérez, 2011).

At present, industrial wastewaters are treated through advanced technologies based on chemical oxidation. Organic contaminants are degraded through the formation of hydroxyl radicals which are extremely reactive and non-selective. These processes have significant drawbacks, most noteworthy being the formation of oxidation intermediates which are even more resilient to degradation. This means that additional energy and chemical reagents are required which renders these processes expensive and economically unfeasible. A promising prospect is the use of advanced oxidation

processes as a pre-treatment step for biological oxidation processes. The aim would be to convert initially toxic and persistent organic components into more suitable biodegradable compounds. It is estimated that a pre-treatment step could significantly lower the costs associated with the overall process. Alternatively, for the case of non-toxic recalcitrant compounds, advanced oxidation processes could be implemented as a post-treatment step. For these cases, the highly biodegradable portion of the wastewater is initially eliminated via a biological process followed by the degrading of recalcitrant pollutants in an advanced oxidation process (Oller, Malato and Sánchez-Pérez, 2011).

3.2.3.2. Industrial wastewater treatment considerations

The properties of industrial wastewaters vary depending on the industry generating them as well as within the specific industry. Compared to domestic wastewater, which generally has a similar composition, the quantitative and qualitative characteristics of industrial wastewater are much more diverse. This results in treatment technologies being complex owing to the large variety of compounds and range of concentrations found in industrial wastewater. For instance, industrial wastewater could contain various other organic substrates which compete with the target pollutant for the oxidising agent resulting in a decrease in process efficiency (Oller, Malato and Sánchez-Pérez, 2011).

Industrial wastewater can be classified into three varieties. Firstly, wastewater comprising of recalcitrant substances such as large macromolecules which are not readily biodegradable owing to their large size and lack of active centres. Secondly, easily biodegradable wastewater containing high quantities of organic substances as well as low concentrations of recalcitrant substances. Finally, wastewater comprising of inhibitory compounds which are toxic to certain microbial cultures. Recently, research (Gogate and Pandit, 2004) and interest into industrial wastewater treatment through biological processes has significantly increased. This is owing to the real likelihood of reusing the wastewater as a safe and valuable water source (Oller, Malato and Sánchez-Pérez, 2011).

The most useful and important biodegradation processes are based on microorganisms such as bacteria and fungi. Important factors to take into consideration when selecting a biodegradation system are the concentration of the species required, their enzyme activity and their ability to acclimatize once they have been exposed to a specific chemical component. Further, microorganisms are very sensitive to environmental changes such as temperature, salinity, pH, oxygen concentration, redox potential, the concentration and types of various substrates and nutrients and the presence of toxic compounds (Oller, Malato and Sánchez-Pérez, 2011).

Most commonly, general measures such as biological oxygen demand (BOD), chemical oxygen demand (COD) and dissolved organic carbon (DOC) are used to characterise industrial wastewater. Furthermore, the ratio of BOD/COD (average oxidation state) provides insight towards the portion of

organic compounds present in wastewater which are biodegradable (Oller, Malato and Sánchez-Pérez, 2011).

3.2.4. Wastewater sources

Availability, cost, carbohydrate content and biodegradability are the main characteristics for the choice of waste materials for use in bio-hydrogen production processes. Photosynthetic hydrogen generating bacteria have been found to be appropriate for the purification of organic waste streams. Favoured substrates for the production of hydrogen include simple sugars such as lactose, sucrose and glucose which are easily biodegradable. Alternatively, pure carbohydrates are also a feasible option for hydrogen production, but are more costly raw materials when compared to simple sugars. The main waste materials which can be used for bio-hydrogen gas production are discussed in this section. Various photosynthetic and fermentative bacteria can make use of waste materials such as municipal solid wastes, industrial effluents and sewage sludge. Both batch experiments and continuous experiments have shown that organic acid containing aqueous streams acquired from such waste was a suitable substrate for the growth of photosynthetic bacteria. These cases were demonstrated using the photosynthetic bacteria *Rhodobacter sphaeroides* which shares many characteristics with *Rhodopseudomonas palustris* (Kapdan and Kargi, 2006).

3.2.4.1. Industrial effluents rich in carbohydrates

Industrial effluents which are non-toxic, rich in carbohydrates and biodegradable such as the dairy industry, olive oil processing industry and winery industry wastewaters can be used as raw substrates for bio-hydrogen gas generation. The above mentioned industrial wastewaters may require pre-treatment steps and processes in order to remove unwanted constituents as well as to ensure an environment suitable for the used bacteria in terms of nutrition. Ideally, high concentration carbohydrate food industry wastewaters should be processed in order to convert the carbohydrate loading to organic acids. Further, the organic acids are then converted to hydrogen gas by means of bioprocessing technologies (Kapdan and Kargi, 2006). Photo-production of hydrogen has also shown to be feasible when utilizing distillery waste water as the substrate. Hydrogen generation from whey by photosynthetic bacteria such as *Rhodobacter capsulatus* and hydrogen production from starch based substrates has also been shown to be possible (Das, 2001).

3.2.4.1.1. Paper mill wastewater

The paper and pulp manufacturing industry is an extremely water intensive industry. In terms of clean water use and withdrawal, this industry is globally ranked third and is only behind the metals and chemical industries. Great quantities of wastewater are generated by the paper and pulp industry as water usage lies between 75 m^3/ton and 227 m^3/ton of product. However, depending on operating

conditions, a certain volume of water is recycled in order to recover fibres which would otherwise be lost in the wastewater. This also effectively decreases the volumes of wastewater produced by this industry (Oller, Malato and Sánchez-Pérez, 2011).

Paper and pulp industry wastewater is known to cause slime growth, thermal impact, scum formation, water colouration and an aesthetically unpleasable environment. Further, the effluent results in an increase in toxic substances in water, which kills zooplankton and fish, and which also has a severe effect on the terrestrial ecosystem. The production of paper based products generate a great number of pollutants which are described by COD, BOD, suspended solids, toxicity and colour. The COD of paper and pulp industry wastewater can be as high as 11 000 mg/L (Oller, Malato and Sánchez-Pérez, 2011).

The most noteworthy pollution sources occur during the wood preparation, pulping, pulp washing, screening, bleaching and paper coating stages of the overall process. The most toxic compounds are produced during the pulp bleaching stage where chlorine is used. In addition, numerous toxic compounds such as resin acids, diterpene alcohols, unsaturated fatty acids, juvaniones and chlorinated resin acids are produced during the pulping process (Pokhrel and Viraraghavan, 2004). However, the composition of the wastewater is largely dependent on the type of process, type of wood used, the technologies applied, management practices, internal recycling of the recovered effluent and the volumes of water used in the process (Oller, Malato and Sánchez-Pérez, 2011).

Currently, effluent treatment methods consist of ozonation, Fenton's reagent, adsorption and membrane technologies. These methods are efficient but uneconomical. Additionally, sedimentation can be used to remove suspended solids and coagulation can be used in order to decrease turbidity (Chen and Horan, 1998; De Pinho *et al*., 2000). Furthermore, adsorption is used in order to reduce COD and adsorbable organic halides (AOX). Despite individual treatment methods improving and cleaning the wastewater to some extent, none of them were able to produce treated effluents which meet the condition of a COD content less than 200 mg/L. Therefore, there is a requirement for alternative processes such as biological treatments which have the potential to degrade the recalcitrant compounds found in paper mill effluents (Oller, Malato and Sánchez-Pérez, 2011).

Current attempts using integrated advanced oxidation/biological systems include an ultrasonic process/activated-sludge bio-treatment, an ozonation/aerobic biological system and an UV-H_2O_2/biological activated carbon for the treatment of raw paper mill wastewaters containing disinfection by-products such as trihalomethanes and haloacetic acids (Oller, Malato and Sánchez-Pérez, 2011).

3.2.4.1.2. Olive mill wastewater

The extraction of olive oils is an agricultural industry with economic importance to South Africa. Olive oil extraction produces large amounts of highly toxic waste which detrimentally impacts plants, soil and aquatic environments and ecosystems (DellaGreca *et al*., 2001). Annual olive mill wastewater generated in the Mediterranean is in excess of 30 000 000 m^3 with the COD content of this effluent as high as 220 000 mg/L. The organic material largely consists of polysaccharides, sugars, polyphenols, polyalcohols, proteins, organic acids and oil. Furthermore, olive mill wastewater contains significant quantities of suspended solids (concentrations up to 190 g/L) (Oller, Malato and Sánchez-Pérez, 2011).

The treatment of olive mill wastewater is complex and multifaceted with no single optimum solution. Presently, the use of advanced oxidation processes is only partially able to decrease the toxicity and enhance the biodegradability of olive mill wastewater. Examples of current treatment methods include TiO2 photo-catalysis and photo-Fenton technologies (Badawy *et al*., 2009). Phenolic compounds can be removed from olive mill wastewater by ozonation, whereas a decrease in phytotoxicity can be achieved through catalytic wet-air oxidation using platinum and ruthenium supported titania or zirconia, and by using a coagulation-flocculation hydrogen peroxide oxidation process (Oller, Malato and Sánchez-Pérez, 2011).

A microbial solution has been proposed for the degradation of both volatile and non-volatile organic compounds found in olive mill wastewater to CO_2 and H_2 (Oller, Malato and Sánchez-Pérez, 2011).

3.2.4.1.3. Winery and distillery wastewater

Wineries and distilleries present a number of obstacles for their wastewater treatment. This industry produces highly rich organic wastewater which is largely dependent on process operating conditions and production activities. The vast majority of effluent, usually above 80 %, is generated during production and generally lasts for 3 months per annum. The wastewater contains sugars, ethanol, organic acids, aldehydes, other microbial fermentation products as well soaps and detergents from clean-up operations. Usually, winery effluent has a low pH (3 – 4) owing to the production of organic acids during natural fermentation, and it typically has relatively large quantities of phosphorous. However, the wastewater is deficient in nitrogen and other trace minerals which are essential for effective biological treatment systems (Oller, Malato and Sánchez-Pérez, 2011).

The COD content of winery effluent is usually between 800 mg/L and 1200 mg/L, however, cases of COD up to 25 000 mg/L have been reported. The COD content is mainly dependent on harvest load and processing activities. Biological treatment systems which have been investigated, for instance activated sludge reactors, are efficient in terms of BOD removal but require extended retention times

(Petruccioli *et al* ., 2000, 2002). Additionally, the capital and operating cost associated with this treatment method are not trivial. As a result, there is no generally accepted and standardized treatment method for winery wastewater (Oller, Malato and Sánchez-Pérez, 2011).

Currently, advanced oxidation processes have shown (Lucas, Peres and Li Puma, 2010) to be promising alternatives for the treatment of this type of wastewater. Specifically, ozonation and ozonation in conjunction with UV-C radiation and/or peroxidation have proven to be efficient in the treatment and biodegradability enhancement of effluents containing polyphenol content such as winery wastewater (Lucas *et al* ., 2010). Treatment by heterogeneous photo-catalysis with TiO2 and homogeneous photo-catalysis with photo-Fenton has also been investigated, making use of UV light during small scale operations and sunlight during larger scale operations. Each of these treatment options has some economic or technical complications and problems. It seems that no single economical solution has been proposed as a complete and comprehensive treatment method for winery wastewater (Oller, Malato and Sánchez-Pérez, 2011).

3.2.4.1.4. Miscellaneous wastewater

Owing to the wide range of polluted industrial wastewaters which are released into the environment, there are some common industrial wastewaters which have barely been investigated in terms of biological treatment methods. An example of such an industry is the leather industry which produces large volumes of highly complex and highly contaminated wastewater. Many water intensive processing stages are involved in transforming raw hides into leather products. Effluents discharged from tanneries contain high concentrations of organic material as well as chemicals such as chlorides, bactericides, emulsifiers, ammonia and detergents. Based on their composition, tannery wastewater cannot be discharged into the environment without pre-treatment due to their toxicity (Vrcek and Bajza, 2001). The nature of tannery wastewater is such that the effluent still exceeds the discharge limits in terms of COD, salinity, ammonia and surfactants once conventional treatment methods have been employed (chromium precipitation, primary sedimentation, biological oxidation and secondary sedimentation). Currently, biological systems are the favoured treatment strategy for tannery wastewaters with advanced oxidation processes considered as a post-treatment step (Oller, Malato and Sánchez-Pérez, 2011).

3.2.4.2. Industrial effluents containing recalcitrant compounds

3.2.4.2.1. Textile wastewater

The textile industry is an extremely large consumer of both water and energy and is responsible for the release of many pollutants which negatively impact the environment. Water consumption in the

range of 80 – 100 m^3/ton of finished textile is consumed. Throughout the textile dyeing and production process, water is used for cleaning the raw material and for many flushing steps. Many different dyes and chemicals are added to water used in textile production, which results in textile effluent having a chemical composition which poses many environmental challenges. Most pollutants are added during dyeing and finishing processes, where finishing incorporates bleaching, dyeing, printing and stiffening of textile products (Oller, Malato and Sánchez-Pérez, 2011).

The main pollutants identified in textile wastewater are suspended solids, highly recalcitrant chemical oxygen demand, dyes giving intense colour and other soluble substances (Dae-Hee *et al*, 1999). The environmental distress lies in the decolourization of textile wastewaters, in the wastewater discharge which contains between 115 – 175 kg COD/ton of finished textile, low biodegradability, high salinity and large range of organic chemicals. Specifically, the pollutants in textile wastewater are accountable for foam formation on the surface of rivers, anomalies in the growth of algae (eutrophication) and toxicity of some aquatic organisms (Oller, Malato and Sánchez-Pérez, 2011).

Usual textile effluents can be characterised by a COD content between 150 mg/L and 12 000 mg/L, total suspended solids between 2900 mg/L and 3100 mg/L, total nitrogen between 70 mg/L and 80 mg/L and BOD between 80 mg/L and 6000 mg/L. This results in a BOD/COD ratio of approximately 0.25 which indicates high quantities of non-biodegradable organic material (Oller, Malato and Sánchez-Pérez, 2011).

In addition to azo dyes, detergent mixtures consisting of non-ionic ethoxylate and anionic aryl sulfonate are also often added in the textile preparation process in order to eradicate impurities from the fabric. Currently, advanced oxidation processes (ozonation, hydrogen peroxide, UV radiation and Fenton's reagent) have been the most popular methods for treating textile wastewater. However, no single unanimous end-of-pipe solution is realizable, and a mixture of processes are required in order to effectively, feasibly and economically treat this wastewater. Based on economic evaluations, biological technologies appear to be the most encouraging (Oller, Malato and Sánchez-Pérez, 2011).

Textiles are given colour through the use of reactive azo dyes which account for approximately 70 % of total dyes produced. Reactive azo dyes are used for their ease of application, high wet fastness profiles, their wide variety of colour shades and for minimal energy consumption. Despite these advantages, approximately 20 % - 50 % of the initial reactive dyes are washed out in the dyeing process. This is owing to the easy hydrolyzation of the dyes and their consequential low affinity for the textile fibres. Therefore, reactive azo dyes remain in the dye bath effluent and are discharged into municipal drainage systems. Effluents containing dyes pose a number of serious problems to both people and

the environment. The effluent streams are aesthetically unpleasing and the split of azo bonds results in aromatic amines which are considered to be carcinogenic and mutagenic (Wang Xingzu, Cheng Xiang, Sun Dezhi, 2007).

In terms of treating textile wastewater for decolourization and detoxification, many physiochemical methods have been tried and tested. These include advanced oxidation processes using the Fenton reagent, hydrogen peroxide or ozone as well as coagulation-flocculation, activated carbon adsorption, membrane filtration, ion exchange, irradiation, and electro-kinetic coagulation. However, these treatments are not realistic or achievable due to their high operating costs and their production of a further undesired waste stream in the form of chemical sludge (Wang Xingzu, Cheng Xiang, Sun Dezhi, 2007).

Conversely, it has been shown (Fu and Viraraghavan, 2001) that azo dyes can be biologically transformed. Initially, White-rot fungus was identified as a promising organism able to degrade a vast variety of recalcitrant compounds by their extracellular enzyme system. However, it has proven challenging to keep the fungus functional in activated sludge systems owing to the unique nutrients and environmental conditions which are required for fungal growth. Further, it has also been shown (Kocyigit and Ugurlu, 2015) that azo dyes can be decolourized by azo reductase under anaerobic conditions which results in the formation of aromatic amines. These aromatic amines can be further biodegraded through hydroxylation and ring-opening (Wang Xingzu, Cheng Xiang, Sun Dezhi, 2007).

Decolourization and mineralization of textile wastewater through a biological process is understood to be an encouraging technology since it is not costly and poses no harm to the environment. Bacteria related to this azo dye bio-decolourization process include the photosynthetic bacteria *R. palustris*. However, a strain may be required with a high decolourization capability (Wang Xingzu, Cheng Xiang, Sun Dezhi, 2007).

In the absence of oxygen, azo bonds in the chromophores can be broken by azoreductase-catalyzed reduction. This results in the bio-decolourization of azo dye effluents. In the microbial electron transfer chain, the azo bond acts as the terminal electron acceptor. Therefore, a readily degradable carbon source is usually necessary. In terms of the decolourization efficiency of Reactive Black 5, an azo dye, glutamine, lactate, butyrate, propionate, acetate, oxalate and formate can be used as co-substrates. These carbon sources are listed from most efficient to least efficient (Wang Xingzu, Cheng Xiang, Sun Dezhi, 2007).

The added nitrogen source also has an effect on the growth and decolourization effectiveness of *R. palustris.* Ammonium chloride has been identified as the most appropriate nitrogen source in terms of growth promotion. Decolourization efficiencies greater than 80 % have been achieved using a variety of nitrogen sources. A noteworthy exception is glutamine, in which the decolourization efficiency was approximately 40 % (Wang Xingzu, Cheng Xiang, Sun Dezhi, 2007).

3.3. Hydrogen

3.3.1. Uses of hydrogen

Hydrogen has many uses. These include as a reactant in hydrogenation processes where hydrogen is used to produce lower molecular weight compounds, saturate compounds, crack hydrocarbons and to remove sulphur and nitrogen compounds. Additionally, hydrogen is used as an O_2 scavenger in order to prevent corrosion and oxidation by chemically removing trace amounts of O_2. Hydrogen is also used as a fuel source for rocket engines and as a coolant in electrical generators (Das, 2001).

Water is the main combustion product of hydrogen (higher heating value of hydrogen is 3042 cals/m^3). Therefore, hydrogen is considered to be a clean and non-polluting fuel which is harmless to both humans and animals. Additional advantageous characteristics of hydrogen are its high conversion efficiency and recyclability (Das, 2001).

3.3.2. Production methods

Presently, hydrogen is primarily produced from water, biomass and fossil fuels. The processes of hydrogen production from water are electrolysis, photolysis, thermochemical methods, direct thermal decomposition and biological production. The processes of hydrogen production from biomass are pyrolysis or gasification. However, these processes generate a combination of gases including H_2, CH_4, CO_2, CO and N_2. The processes of hydrogen production from fossil fuels are steam reforming of natural gas, thermal cracking of natural gas, coal gasification and partial oxidation of heavier than naphtha hydrocarbons. From the aforementioned methods, approximately 90 % of hydrogen is generated by steam reforming processes where natural gas or light oil fractions are allowed to react with high temperature steam. Additional procedures for hydrogen production on an industrial scale include electrolysis of water and coal gasification. A drawback of these industrial processes is that they mostly rely on the consumption of fossil fuels as an energy source. Hydrogen production through electrochemical and thermochemical methods is also energy intensive and largely environmentally unfriendly. Alternatively, hydrogen produced through biological processes are largely operated at ambient pressures and temperatures resulting in less energy intensive processes. Biological hydrogen production methods are not harmful to the environment and have an additional benefit of leading to

new technologies for the utilization of inexhaustible renewable energy resources. Additionally, biological processes utilize waste streams which aids waste reuse and recycling (Das, 2001).

3.3.3. Biological hydrogen production processes

Hydrogen produced through biological processes can be grouped as follows:

1. Utilising cyanobacteria and algae for the bio-photolysis of water.
2. Utilising photosynthetic bacteria for the photodecomposition of organic compounds.
3. Fermentative hydrogen production from organic compounds.
4. Combination systems utilising both photosynthetic and fermentative bacteria.

The most encouraging microbial system for biological hydrogen generation has been identified as phototrophic bacteria. The main advantages include high theoretical conversion yields of organic material to hydrogen gas, the capacity to use wide spectrum of light, the ability to consume waste streams as organic substrates, subsequent applications in wastewater treatment and the lack of oxygen evolving activity. A lack of oxygen evolving activity is desired as the presence of oxygen leads to the inactivation of various microbial systems. Of particular interest is the ability of phototrophic bacteria to consume organic substrates present in industrial wastewater streams. (Das, 2001). Photosynthetic bacteria produce hydrogen from organic acids in a light dependent reaction known as photo-fermentation, thereby generating hydrogen from substrates which is normally difficult from a thermodynamic standpoint. This is possible through the input of additional energy. Additional energy can be sourced through bacterial photosynthesis. In the past, waste streams containing organic acids have been used as substrates, possibly with the added benefit of waste treatment. Currently, a variety of novel substrates have been identified as effective in a photo-fermentative process, thereby extending the scope of these bacteria. The characteristics of this process allow for virtually stoichiometric conversions of the various substrates to hydrogen. However, many noteworthy problems and obstacles remain. These include low light conversion efficiencies, as well as the high energy demand and low turnover number of nitrogenase. Further, expensive photo-bioreactors are potentially required (Hallenbeck and Liu, 2016).

3.3.3.1 Basic mechanisms

The nitrogenase enzyme is responsible for the production of hydrogen and is active during N_2 reduction, which reduces protons to hydrogen. This occurs during the absence of nitrogen. This reaction requires energy and is therefore dependent on ATP. Typically, photosynthesis is able to provide the additional energy through the production of ATP via the light driven creation of a proton gradient. Low potential electrons, necessary for proton reduction, are generated by the proton

gradient by means of a reverse electron flow process. The nitrogenase enzyme is inactive under nitrogen rich conditions, so usually substrates are required to be nitrogen poor for the production of hydrogen. Hydrogen production rates and yields can be improved through an understanding of the metabolic pathways involved. By means of metabolic engineering, an increase in the reduction of protons to hydrogen by nitrogenase can be achieved. This is done by blocking pathways which divert NADH, CO_2 fixation and polyhydroxybutyrate (PHB) synthesis (Hallenbeck and Liu, 2016).

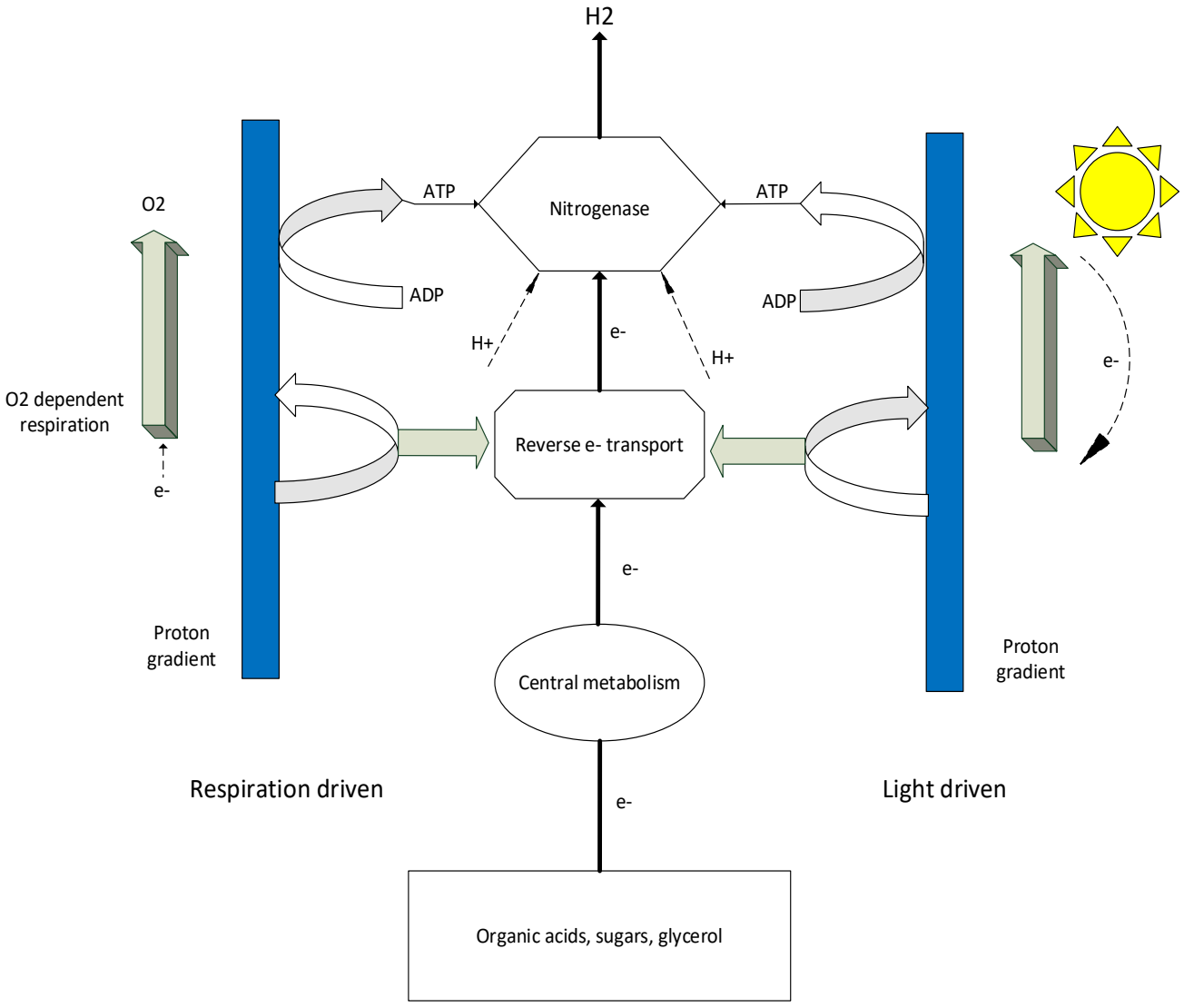

Figure 2: *Integration of H_2 production by photosynthetic bacteria with cellular metabolism (Hallenbeck and Liu, 2016).*

Figure 2 shows a diagram of the interactions with the differing photosynthetic bacterial metabolisms required for nitrogenase activity. ATP can either be provided by oxidative phosphorylation during micro aerobic respiration (left) or by cyclic photophosphorylation (right). The central metabolism is responsible for the generation of high energy electrons. This occurs through either reverse electron flow driven by proton gradients created during micro aerobic respiration (left) or by cyclic photosynthesis (right) (Hallenbeck and Liu, 2016).

The following equations show the theoretical photo-conversion of organic compounds. Equation 6 is for acetic acid and equation 7 is for glucose (Pintucci *et al* ., 2015).

$$C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad [6]$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \quad [7]$$

There may either be a surplus or shortage of chemical energy depending on the organic substrate used. However, light is the main source of energy which drives the conversion and which is responsible for the rate at which the conversion takes place. The light is stored as energy in ATP which is formed by photoelectron transport, phosphorylation or a combination of the two. *R. palustris* is considered a candidate organism for studying the role of phosphorylation in metabolic flexibility and regulation. This is owing to the ability of *R. palustris* to biodegrade various organic substrates and sources in differing environmental conditions. Since, *R. palustris* is capable of both biodegradation and bioenergy production, an anaerobic photoheterotrophic is most suitable(Pintucci *et al* ., 2015).

Given suitable conditions, such as an anaerobic environment in the illumination of light, certain photo-heterotrophic bacteria are able to produce hydrogen gas and carbon dioxide gas through the conversion of organic acids such as acetic acid, butyric acid and lactic acid. A good source of organic acids which can be converted to hydrogen gas through anaerobic photosynthetic bacteria are those produced during the acidogenic step of organic waste anaerobic digestion processes (Kapdan and Kargi, 2006).

The optimal temperature for growth of photosynthetic bacteria is between 30 ◦C and 35 ◦C whereas the optimal pH of photosynthetic bacteria is at a pH of 7. Photosynthetic bacteria favour organic acids to carbohydrates as a source of carbon. Specifically, acetic acid, butyric acid, propionic acid, lactic acid and malic acid have been reported as preferred carbon sources. Nonetheless, complex carbohydrates and appropriate industrial effluents are also useful for hydrogen gas production by photosynthetic organisms (Kapdan and Kargi, 2006).

Table10 in Appendix A summarizes the rates of hydrogen gas production from various carbon containing components such as organic acids and carbohydrates as well as by various photo-fermentative bacteria. Initially, Table10 is arranged according to carbon containing components, but this switches to the industry from which the wastewater was obtained when more than one carbon containing component is present. Hydrogen production rates are dependent on many factors such as illumination intensity, carbon source and the type of photo-fermentative organism used (Kapdan and Kargi, 2006).

3.3.4 Major enzymes

Photosynthetic bacteria contain three distinct enzymes which are able to produce and metabolize hydrogen. These are the reversible hydrogenase enzymes, the uptake hydrogenase enzymes and the nitrogenase enzymes (Das, 2001).

3.3.4.1. The reversible hydrogenase enzymes

Ferredoxin and other low redox electron carriers, which can either be artificial or natural, are oxidised by the reversible hydrogenase enzymes. The oxidation reaction is a readily reversible reaction (Das, 2001).

3.3.4.2. The uptake hydrogenase enzymes

Given low partial pressures, the uptake hydrogenase enzymes are able to reduce a comparatively high-potential electron acceptor resulting in the enzymes taking up hydrogen. However, this process generates negligible quantifiable hydrogen. As the hydrogenase enzyme utilizes hydrogen gas, its activity counteracts nitrogenase activity and should therefore be avoided and limited for improved hydrogen gas evolution. Manipulated cultures of photosynthetic bacteria which are hydrogenase deficient are able to generate even greater volumes of hydrogen gas (Das, 2001).

3.3.4.3. The nitrogenase enzymes

The nitrogenase enzymes are able to produce hydrogen when N_2 gas is absent. When N_2 gas is present, the nitrogenase enzymes convert the N_2 gas to ammonia. The evolution of hydrogen by the nitrogenase enzymes is an energy intensive irreversible reaction due to the reaction being linked to the hydrolysis of a minimum of four ATP per H_2 generated. It is possible for bacteria to have any combination of the abovementioned enzymes present and active at the same time. The activity of the enzymes can switch drastically and is dependent on growth and culture conditions. Additionally, there are also various types of each enzyme which can be differentiated based on the metal content and which can be present in the same organism concurrently. The complexity of the enzymes can result in moderate difficulty when the net hydrogen generation or uptake is investigated and analysed (Das, 2001).

The most important and key enzyme for photosynthetic bacteria, in terms of hydrogen gas evolution, is the nitrogenase enzyme. The action of this enzyme is dependent on the presence of ammonia and oxygen as well as the ratio of nitrogen to carbon. Inhibitory activity is observed at high nitrogen to carbon ratios, and when ammonia and oxygen are present. Therefore, optimum process conditions are anaerobic with ammonia limitation. Ideally, glutamate and yeast extract should be used as the nitrogen source for improved hydrogen gas evolution, with ammonia salts avoided. For the case of

high nitrogen concentrations, the metabolic activity of the photosynthetic bacteria shifts towards utilizing the carbon substrate for cell synthesis instead of hydrogen gas evolution. This results in surplus biomass growth which decreases light penetration to the culture system (Kapdan and Kargi, 2006).

3.3.5. Conditions for photo-fermentation

3.3.5.1. Illumination intensity

Photo-fermentation of organic substrates is dependent on light intensity. In addition, certain organic acids might require greater illumination intensities in order to be broken down. As the light intensity increases, both the hydrogen generation rate and the hydrogen yield will increase, but the light conversion efficiency will decrease (Kapdan and Kargi, 2006). Photosynthetic bacteria are able to utilize different wavelengths of the electromagnetic spectrum depending on the variation of photosynthetic bacteria as well as on the variation of photosynthetic pigments (carotenoids and chlorophylls), thereby allowing them to live in a variety of different environments.

3.3.6. Different approaches toward improving H_2 production by photosynthetic bacteria

Various methods can be employed in order to improve both hydrogen production rates and hydrogen yields by photosynthetic bacteria. These are shown in Figure 3 and include improved photo-bioreactor design which allow for increased light penetration, the blocking of competing metabolic pathways and the optimization of experimental processes which both allow for improved substrate utilization (Hallenbeck and Liu, 2016).

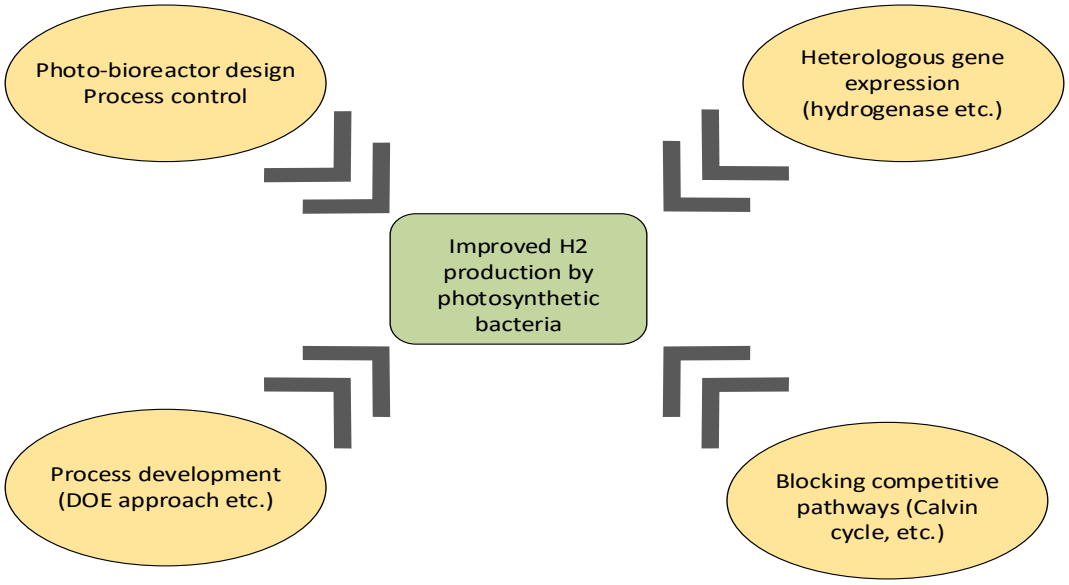

Figure *3: Various approaches toward improving H_2 production by photosynthetic bacteria.* Figure *adapted from (Hallenbeck and Liu, 2016).*

3.3.6.1. Photo-fermentation

The vast majority of research into photosynthetic bacterial hydrogen production has involved the utilization of organic acids as substrates such as lactate, acetate, and succinate. These bio-reactions

only occur when no ammonium is present. The reduction of protons to hydrogen under these conditions is necessary in order to sustain a balanced intracellular oxidation-reduction potential despite the energy intensive CO_2 fixation in the presence of a readily assimilated fixed carbon source (Hallenbeck and Liu, 2016).

Recent investigations reveal that the formation of soluble microbial products (SMP's) are also contributing to reduced hydrogen yields. However, this problem can be solved through the addition of ethanol to the process. It must be noted that the mechanisms of this effect are not fully understood. A reduction in the production of soluble microbial products is not an obvious metabolic process, since the soluble microbial products produced during fermentation are comprised of a complex mixture of proteins, amino acids, nucleic acids, organic acids, products of energy metabolism and cellular structural components (Hallenbeck and Liu, 2016).

3.3.6.2. Single and two-stage systems

A number of systems have been examined where fermentative bacteria are used together with photosynthetic bacteria in order to achieve almost complete conversion of substrates to hydrogen. Theoretically, when glucose is used as a substrate 12 mol of H_2 can be produced from 1 mol of glucose. This reaction is shown in equation 8 (Hallenbeck and Liu, 2016).

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \qquad [8]$$

For the case of dark fermentation, a maximum of 4 mol of H_2 can be produced which is a yield of only 33 %. Additionally, side products, mainly organic acids, are produced during dark fermentation. These organic acids, such as acetate and lactate, are useful substrates for use in photo-fermentation processes which are able to generate greater hydrogen yields (Hallenbeck and Liu, 2016).

A single combined dark/light process or a two stage process (dark followed by light) can be used to convert organic acids to supplementary hydrogen gas using photosynthetic bacteria. Despite being theoretically feasible, practically these processes are challenging to control and optimize. Disadvantages of combined systems include the contrasting culture necessities of the two dissimilar organisms, the low efficiency and molar hydrogen yields of the overall process as well as the expensive treatment required for dark fermentation effluent which can contain inhibitory compounds to photosynthetic bacteria. A further drawback of combined systems is the complex bio-reactor design required for the two dissimilar processes (Hallenbeck and Liu, 2016).

Therefore, it seems that a simple single stage process is preferred owing to the virtually stoichiometric conversion of substrates to hydrogen and the effective and efficient nature of the process.

Furthermore, direct photo-fermentation of more complex carbon substrates such as sugars and carbohydrates to hydrogen has recently proven to be successful. Despite the preferential use of organic acids as substrates for photosynthetic bacteria, research (Keskin, Abo-Hashesh and Hallenbeck, 2011) has shown that sugars can also be used. Sugars are also abundantly found in various industrial waste streams. It has been shown that hydrogen production by photosynthetic bacterium *Rhodobacter capsulatus JP91* is possible using glucose as substrate. However, a moderately low H_2 yield was achieved (3 mol H_2 per mole of glucose). In terms of waste streams, appropriately diluted sugar refinery wastes (beet and blackstrap molasses) were converted to hydrogen in a single photo-fermentation process. Finally, biodiesel industry waste in the form of glycerol, has been converted to hydrogen through photo-fermentation (equation 9). This process achieved a yield of 6.9 mol H_2 per mole of crude glycerol which corresponds to a yield of 98 % (Hallenbeck and Liu, 2016).

$$C_3H_8O_3 + 3H_2O \rightarrow 7H_2 + 3CO_2 \qquad [9]$$

3.3.6.3. Micro-aerobic hydrogen production

Additional energy is required in order to achieve a hydrogen yield greater than 4 mol per mol glucose. The additional energy is provided by captured photons during photo-fermentation. However, this process is only moderately efficient owing to the requirement of consistent light as well as the inherent inefficiency of photosynthesis. To counteract this and to add metabolic energy to the process, micro-aerobic respiration is a noteworthy possibility. Micro-aerobic respiration would result in extra ATP and proton gradients which are required to drive the reverse electron flow necessary to produce the reductant for nitrogenase activity. However, this seems contradictory as hydrogen production is only possible during anaerobic processes. Nevertheless, micro-aerobic nitrogen fixation can be achieved by certain photosynthetic bacteria. Finally, hydrogen yields can be increased through optimization of micro-aerobic respiration. If oxygen can be added to bioprocess systems in a precise and accurate manner, then additional energy can be generated without wastefully oxidising the substrate. Oxygen concentrations should also be low enough that nitrogenase activity is not inhibited (Hallenbeck and Liu, 2016).

3.4. Cell immobilization

The practice of bioprocesses using cell immobilization methods has gained substantial interest and attention recently. Specifically, cell immobilization in polyvinyl alcohol (PVA) cryogels has become attractive due to the extensive variety of biotechnological and biomedical applications of the cryogels. These include use as biocatalysts, drug delivery systems, chromatographic materials as well as cell and enzyme transport and growth mediums. In particular, PVA cryogels can be used to immobilize

photosynthetic bacteria, since cryogels can be produced which are biocompatible, mechanically and chemically stable and transparent.

Additionally, immobilization also offers a sheltered and protected environment for cells compared to free cells in suspension within a bioreactor. Additionally, it is desired to create optimal conditions of thermal and mechanical stability as well as a suitable porosity for the cryogels. The cryogels need to be permeable enough so that the ingress of nutrients needed by the organisms as well as the possible products produced by the cells can discharge and diffuse through the beads. Preferably, the pore size should be adequate so as to not limit mass transfer into and out of the cryogel. These products can then be collected and utilized accordingly (Szczsna-Antczak & Galas 2001). Figure 4 shows cells immobilized in a PVA cryogel.

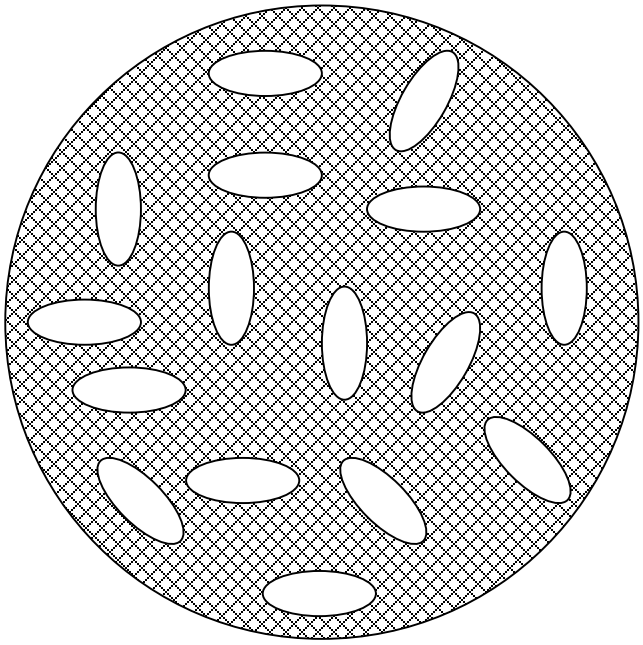

Figure *4: Cell immobilized in PVA cryogel.*

3.4.1. Characteristics of PVA

PVA is a colourless, water soluble synthetic resin polymer which is non-toxic to organisms and which can be economically manufactured on an industrial scale. Selected physical properties of PVA can be seen in Table 13 in Appendix F (Tao 2003). The PVA monomer, which is a repeating unit forms a large molecule known as a polymer, with chemical formula $[CH_2CH(OH)]_n$. The structural formula of PVA can be seen in Figure 5.

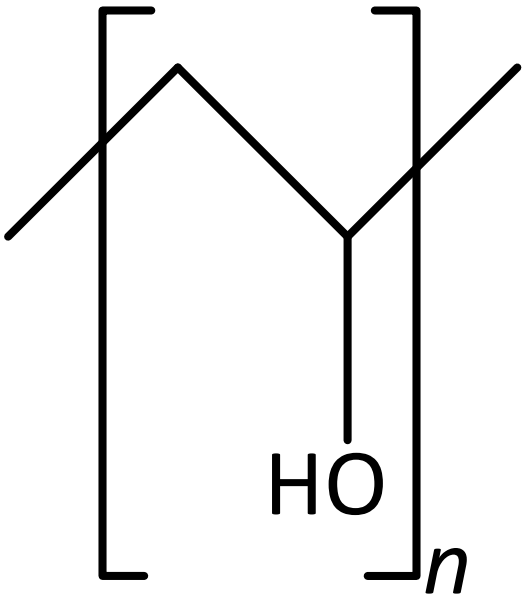

Figure *5: Structural formula of PVA.*

PVA is of great interest, particularly in the pharmaceutical and biomedical fields owing to its characteristics and properties. Specifically, the crystalline nature of PVA which results in physically cross-linked cryogels when produced from the freezing thawing method is of interest. Additionally, industrially manufactured PVA is obtainable which is fully hydrolyzed. Hydrolyzed refers to the conversion of polyvinyl acetate to polyvinyl alcohol. The degrees of hydrolysis are commonly above 98.5 % (Hassan and Peppas, 2000).

3.4.2. PVA cryogels

Hydrogels comprise of a system of cross-linked polymers which are hydrophilic, meaning they can dissolve more easily in water (in comparison to organic solvents) as they are able to form hydrogen bonds. Hydrogels swell when they are exposed to organic liquids and water, however, they are able to remain insoluble and do not dissolve in solution due to their structural characteristics and cross-linked system. The cross-linked configuration of PVA makes them especially valuable, advantageous and useful as materials such as hydrogels. When hydrogels are produced using extremely cold temperatures they are referred to as cryogels (with cryo defined as something which encompasses extreme cold) (Hassan and Peppas, 2000).

The production of cross-links within the PVA cryogel can be enhanced with the adding of crosslinking agents which belong to the monoaldehydes (such as acetaldehyde and formaldehyde). Throughout the crosslinking procedure, high concentrations of these crosslinking compounds are found in the PVA cryogels which kill immobilized organisms. Additionally, tiny quantities of the crosslinking compounds stay in the PVA cryogels resulting in them being incompatible for biological, pharmaceutical and biomedical applications. The harmful crosslinking compounds have the potential to affect the

biological activity of the cells or enzymes which results in the degradation of biologically active cells. In order to eliminate the crosslinking compounds from the PVA cryogels, a tedious and time consuming removal needs to be conducted which results in this cross-link formation procedure being infeasible (Hassan and Peppas, 2000).

Another possible technique for crosslink development includes the use of electron beam or gamma-irradiation of aqueous PVA solutions, which has an improvement over the use of crosslinking compounds, in that it does not leave harmful and toxic residues within the PVA cryogel. However, a disadvantage recognized in this technique of crosslinking, is bubble formation which is only known to be solved by chilling the aqueous PVA solution before cryogel production, as this yields uniform cryogels. This is not endorsed when using PVA and adds further obstacles to the overall cryogel process (Hassan and Peppas, 2000).

The crosslinking system within the PVA cryogels can also be produced by means of a physical technique which does not need the use of crosslinking compounds. This is crucial, as the use of crosslinking compounds is harmful to immobilized cells and eventually results in the death of the cells. The physical technique used to from the crosslinking system is exposing the PVA solution to tremendously cold temperatures (for instance liquid nitrogen) and then allowing the PVA to thaw, upon which aqueous PVA solutions exhibit the properties of crystallite formation. The stability as well as the number of the crystallites which are formed increases as the number of freezing and thawing cycles is increased. Crosslinking by crystallite formation results in increased mechanical strength when compared to cryogels cross-linked by chemical or irradiative approaches (Hassan and Peppas, 2000).

Furthermore, the cryogel characteristics, for the physically cross-linked technique, are also reliant on the molecular weight of the PVA solutions, the concentration of the PVA solutions, the temperature and period of freezing, the rate of thawing as well as the number of freezing and thawing cycles.

3.4.3. PVA cryogel crystallization

The crystalline configuration of PVA, consisting of crystallites of PVA, can be viewed on a molecular level as a layered arrangement whereby the molecules are held together by hydroxyl bonds (an oxygen atom covalently bonded to a hydrogen atom). The bonds between the structured layers are van der Waals forces which are not as strong as the hydroxyl bonds. Additionally, the magnitude of the crystallites increases as the PVA polymer molecular weight increases, while the appearance of the crystallites are independent of the PVA polymer molecular weight (Hassan and Peppas, 2000).

The glass transition temperature, the temperature range in which a polymer material changes from a hard and glassy material to a soft and elastic material, of non-aqueous PVA is determined to be 85 °C.

However, the glass changeover temperature of aqueous PVA solutions is known to be considerably less than that of dry PVA. The gelation point of PVA solutions is expressed as an degree of chemical reaction and can be regarded as a temperature threshold for the development of a thermo-reversible gel. Thermo-reversible gels such as cryogels are formed throughout the chilling of the polymer solution which takes place during the freezing and thawing process. At the gel point, a solid system of crosslinking polymer chains are produced and this is the maximum temperature at which the occurrence of a network is observed (Hassan and Peppas, 2000). A cross-linked network is preferred as this offers an organized and stable medium for the immobilization of cells.

3.5. Important conclusions from literature study

It is expected that industrial wastewater effluents will vary greatly with respect to their composition and the volumes produced. This will make the treatment of these wastewaters by photo-fermentation challenging as a degree of inconsistency and uncertainty is associated with each wastewater. However, many of the industrial wastewaters contain large concentrations of highly biodegradable organic material which are ideal substrates for growth of *R. palustris* and from which photo-fermentative treatment is possible. This treatment also has the potential to replace the current wastewater treatment methods for certain wastewaters, particularly those which use or produce compounds which are toxic, biologically harmful or recalcitrant. Finally, many of the industrial wastewaters contain nitrogenous compounds which will inhibit nitrogenase activity as well as hydrogen gas production.

4. METHODOLOGY

This section outlines the approach taken in conducting the industrial wastewater survey of South African industries and lists the effluent samples obtained from the various industries. The criteria and conditions for photo-fermentative treatment and hydrogen production are also established and outlined. Further, the experimental setup is described and the experimental procedure and conditions for bacterial growth and wastewater treatment are explained. Finally, the procedure used to immobilize the *R. palustris* cells is outlined.

4.1. Wastewater survey

A three-step elimination procedure was applied to each identified industrial wastewater. Firstly, the viability of photo-fermentative wastewater treatment for each industry was assessed. The assessment of photo-fermentative treatment suitability was made by analyzing the wastewater components. Those containing organic material such as sugars, organic acids, carbohydrates, starches and proteins were deemed to have good photo-fermentative treatment potential whereas those containing substances considered harmful to *R. palustris* such as heavy metals, caustic detergents, strong acids and alkalis and inorganic compounds were deemed to have poor photo-fermentative treatment potential. Secondly, the degree to which each industrial wastewater supports microbial growth was evaluated. Finally, the wastewater treatment potential of *R. palustris* was examined by monitoring the decrease in contaminant levels characterized by chemical oxygen demand (COD) and the hydrogen production potential of *R. palustris* was investigated.

The survey identified nine South African industries which produce organic containing wastewater. Wastewater from several industries has been obtained.

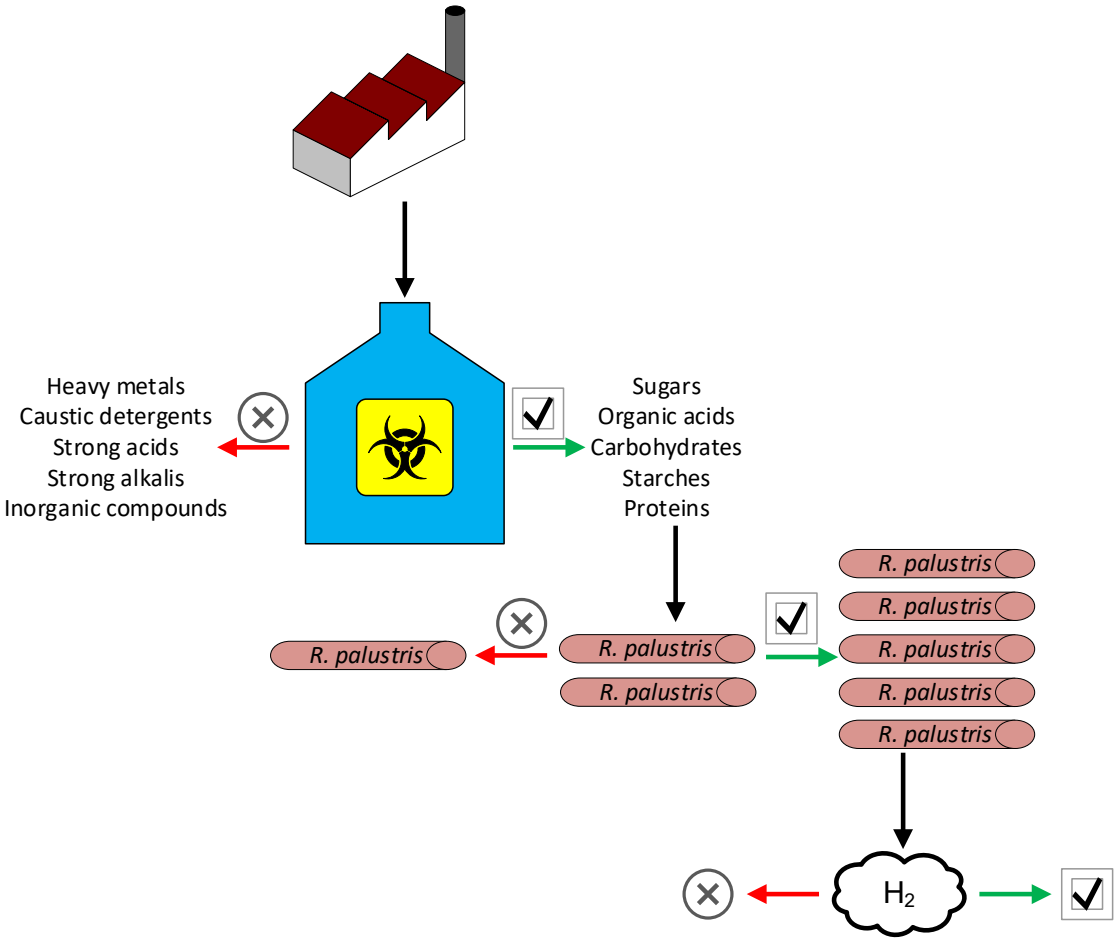

Figure *6: Wastewater viability process.*

4.2. Wastewater

The industrial wastewater effluents which have been collected for this research are described in this section.

4.2.1. Winery wastewater

Winery wastewater was supplied by a wine producer located near Paarl, South Africa. Two fresh 5L samples were obtained and rapidly frozen in a deep freezer (-10 °C). The first sample consisted of winery processing water before any treatment methods were employed. It is estimated that the COD of this water is approximately 12 000 mg/L, as stated by an on-site employee of the wine producer. The second sample comprised of winery wastewater after a treatment step which takes the form of a settling tank. It is estimated that the COD of the water after the settling tank is approximately 4000 mg/L, as stated by an on-site employee of the wine producer. The wine producer estimates that they use approximately 12 000 000 L/month of water of which 70 % ends up as wastewater. The COD content of their wastewater is monitored by the municipality.

4.2.2. Anaerobic digester effluent

Anaerobic digester effluent was supplied by a master's student at Stellenbosch University. The anaerobic digester was fed with fruit waste and manure. One fresh 5L sample was obtained and rapidly frozen in a deep freezer (-10 °C). It is estimated that this stream has a high loading of organic acids, as stated by the Stellenbosch University master's student.

4.2.3. Brewery wastewater

Brewery wastewater was supplied by a master's student at Stellenbosch University. The master's student supplied brewers' spent grains (BSG) which were mechanically pressed to produce a liquid containing sugars, starch (polysaccharides) and proteins. One fresh 5L sample was obtained and rapidly frozen in a deep freezer (-10 °C).

4.2.4. Sugar processing wastewater

Vinasse was supplied by a South African sugar producer. Two fresh 5L samples were obtained and rapidly frozen in a deep freezer (-10 °C).

4.2.5. Olive mill wastewater

Olive mill wastewater was supplied by an olive oil producer located near Paarl, South Africa. Two fresh 5L samples were obtained and rapidly frozen in a deep freezer (-10 °C). The first sample consisted of olive processing water from a separation process whereby olive oil is separated from solid material. The second sample comprised of the overall wastewater from the plant, including water containing cleaning agents, etc. The olive oil producer estimates that they discharge 1500 L/day of wastewater into the ground. They also have no current wastewater treatment methods and it is not monitored.

4.2.6. Tannery wastewater

Tannery wastewater was supplied by a leather wholesaler located near Wellington, South Africa. One fresh 5L sample was obtained and rapidly frozen in a deep freezer (-10 °C). The sample consisted of the overall tannery processing water from the plant, including water containing cleaning agents, etc. The leather wholesaler estimates that the COD of this wastewater lies between 3000 mg/L and 6000 mg/L depending on process operating conditions. They discharge their wastewater into a dam located near the plant and have no current wastewater treatment methods. The COD content of their wastewater is monitored by the municipality.

4.2.7. Fish processing wastewater

Fish processing wastewater was supplied by a leading fishing company located in Woodstock, Cape Town. Two fresh 5L samples were obtained and rapidly frozen in a deep freezer (-10 °C). The first sample consisted of fish processing wastewater. It is expected that this water has high protein and organic content. The second sample comprised of ice melt wastewater. It is expected that this wastewater will have minimal to no organic content and have a high concentration of salt. The fishing company estimate that they discharge 900 kL/day of wastewater.

4.2.8. Paper and pulp mill wastewater

Paper and pulp mill wastewater was supplied by a pulp and paper company located near Durban, South Africa. Two fresh 5L samples were obtained and rapidly frozen in a deep freezer (-10 °C). The first sample consisted of entering clarifier effluent whereas the second sample consisted of paper machine effluent.

4.2.9. Textile dye wastewater

Textile dye wastewater was supplied by a dye house located in Observatory, Cape Town. One fresh 5L sample was obtained and rapidly frozen in a deep freezer (-10 °C). The sample consisted of three dye types. These include Yellow 3RS, Red 3B5 and Navy. The dye house estimates that they discharge 1100 kL/month of wastewater into municipal sewers. They rely on dilution from other process wastewater streams in order to get their solvent, chloride and chemical oxygen demand (COD) levels into an acceptable range. These levels are monitored by the city council.

4.2.10. Wastewater procurement summary

Table 3 shows the specific wastewater stream obtained from each industry for experimental use.

Table 3: Specific waste streams obtained from local industries. The sources have been anonymized.

Industry	Specific waste stream
Winery	Winery processing water.
AD	AD effluent (AD fed with fruit waste and manure)
Brewing	Liquid pressing from spent brewing grains.
Sugar processing	Vinasse
Olive processing	Overall process wastewater.
Tanning and leather finishing	Overall tannery process wastewater.
Fish processing	Fish processing wastewater.
Paper and pulp	Effluent from paper machine
Textile dye	Dye wastewater containing Yellow 3RS, Red 3B5 and Navy.
Fruit processing	Anaerobic digester (AD) effluent. AD fed with apples, pomace and cow manure.

4.3. Experimental setup

The experimental setup for hydrogen evolution can be seen in Figure 7. *R. palustris* was grown in a simple photo-bioreactor (6) which was located in a temperature controlled hot water bath (5). Light illumination was provided by a series of 100 W incandescent light bulbs (1). Illumination to the entire microbial culture was ensured through magnetic stirrers (7). Two sample ports were located on the photo-bioreactor, one for liquid sampling and potential liquid additions (for instance nutrients and substrates) (4) and one for gas sampling (2). Furthermore, these sample ports were used to sparge sterile argon into the photo-bioreactor which created the desired oxygen and nitrogen free conditions. The evolved hydrogen gas was trapped in a graduated plastic cylinder (3). The temperature of the water in the water bath was controlled by means of a heating circulator which was set at an experimental temperature (8). Heat was added to the water through radiation from the light bulbs. Heat was removed from the water by circulating it through a tank of water located in a fridge (10). Additional heat was supplied by the heating circulator. (Pott, 2013).

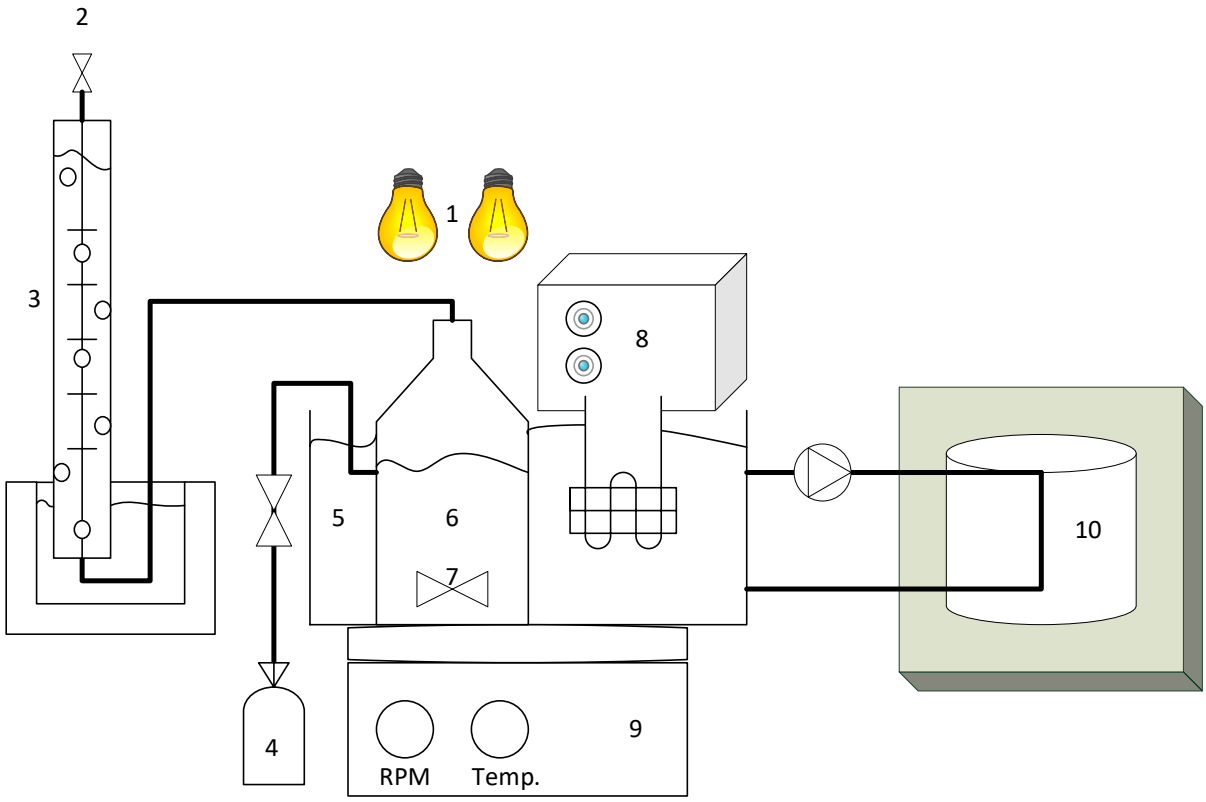

Figure *7: Schematic diagram of photo-bioreactor experimental setup: (1) incandescent light bulbs; (2) gas sampling port; (3) graduated plastic cylinder; (4) liquid sampling port; (5) temperature controlled hot water bath; (6) photo-bioreactor; (7) magnetic stirrer; (8) temperature control; (9) agitation control; (10) cooling system.*

4.4. Experimental procedure

4.4.1 Media

Cultures of *R. palustris* were grown in a *Rhodospirillaceae* minimal media. In order to make a 1 L stock solution of media, stock solutions of K_2HPO_4 (17 g/100 ml), KH_2PO_4 (17 g/100 ml), bulk nutrients and trace elements were made. Bulk nutrients and trace elements contained (per litre):

Bulk nutrients (g)	Trace elements (mg)
2 g yeast extract	70 mg $ZnCl_2$
1.6 g $Na_2S_2O_3$	100 mg $MnCl_2{\cdot}4H_2O$
0.02 g PABA	60 mg H_3BO_3
2 g $MgSO_4{\cdot}7H_2O$	200 mg $CaCl_2{\cdot}6H_2O$
0.5 g $CaCl_2{\cdot}2H_2O$	20 mg$CuCl_2{\cdot}2H_2O$
4 g NaCl	20 mg $NiCl_2{\cdot}6H_2O$
0.05 g Ferric citrate	40 mg $NaMoO_4{\cdot}2H_2O$

A total volume of 982 ml was made containing 10 ml K_2HPO_4, 10 ml KH_2PO_4, 100 ml of bulk nutrients solution and 862 ml distilled water. This solution was autoclaved at 121 °C for 5 minutes. Finally, 1 ml of trace elements, 1 ml of vitamin B12, 1 ml of thiamine, 10 ml of glycerol (5M) and 5 ml of glutamate (2M) were added as sterile additions to bring the final volume of the media to 1 L.

The glycerol (5M) and glutamate (2M) were prepared by mixing the correct ratios of glycerol and glutamate with distilled water. Sample calculations for these procedures can be seen in Appendix G.

4.4.2. Analytical procedures

4.4.2.1. Wastewater preparation

Each wastewater sample was centrifuged at 10 000 RCF for 20 minutes prior to sterilization in an autoclave at 121 °C for 15 minutes.

4.4.2.2. Cell dry weight determination

Each liquid sample was centrifuged at 14 500 RPM for 5 minutes. The supernatant is removed for further analysis and the cell pellet is suspended in an equal volume (as the supernatant) of phosphate-buffered saline (PBS). The optical density (OD) of the suspended sample was measured in a spectrophotometer at 660 nm where the absorbance was correlated to cell dry weight according to equation 10 (Pott, 2013).

$$cdw\ \left(\frac{g}{L}\right) = 0.39(OD_{660\,nm}) \qquad \text{[Equation 10]}$$

4.4.2.3. COD concentration

The COD concentration was determined through a colorimetric analysis. Each sample was added to an oxidizing chromatic acid solution and allowed to react for 2 hours at 148 °C in a Spectroquant Thermal Reactor 420 followed by the reading of the COD concentration from a Spectroquant Prove spectrophotometer.

4.4.3. Growth and hydrogen production

R. palustris was grown heterotrophically, organotrophically and phototrophically. This means carbon was obtained from organic compounds, reducing equivalents were also obtained from organic compounds and energy was obtained from light. This classifies *R. palustris* as a photoorganoheterotroph under these conditions. When grown in *Rhodospirillaceae* minimal media, virtually all H_2 was produced not as a by-product of NADH metabolism but rather as a by-product of the nitrogen fixing cycle mediated by nitrogenase

4.4.4. Step by step experimental procedure

The step by step experimental procedure for the growth experiments and wastewater treatment tests is given in this section. Firstly, the wastewater samples were prepared according to the method given in section 4.4.2.1. Media was prepared according to the procedure given in section 4.4.1. Prior to inoculation the following equipment was autoclaved: bioreactors, magnetic stirrers and pipette tips. Further, so as to ensure a sterile environment, the bioreactors were prepared and inoculated in a fume hood. The experimental volumes of wastewater and media were added to the bioreactors. Next the bioreactors were inoculated from a liquid culture grown on the media described in section 4.4.1 so that the initial OD of the bioreactor volume was less than 0.05. Finally, the bioreactors were sparged with Argon gas for 5 minutes at a flowrate of 40 L/min. The bioreactors were then placed in the hot water bath and connected to the graduated cylinders. Samples (approximately 5 ml) were withdrawn from the bioreactors by a syringe three times a week for cell dry weight determination and COD concentration measurements. Cell dry weight was determined according to the procedure in section 4.4.2.2. and COD concentration was determined according to the method given in section 4.4.2.3. Gas samples were taken on the final day of the experimental duration by a syringe. The gas samples were then injected into gas chromatography equipment where the composition was determined.

4.5. Immobilization

The procedure for the immobilization of *R. palustris* in PVA cryogel beads is given below. Prior to immobilization, the following equipment was autoclaved: pipette tips, sieve, plastic Tupperware, peristaltic pump tubes, multiple outlet tube, Falcon tubes.

Firstly, PVA solutions (10 % PVA by mass in an equal mixture of distilled water and reagent grade glycerol) were prepared using PVA with a molecular weight of 78 000 g/mol. 500 g PVA solutions were prepared in Schott bottles by dissolving 50 g of PVA in 450 g of distilled water and glycerol (225 g distilled water and 225 g glycerol). Mixing and heating (solution temperature approximately 90 °C with the hotplate set to 150 °C) was applied to the PVA solutions until a homogeneous phase was present. The exact duration of heating and mixing was not recorded, however, the duration of the process was approximately 3 hours. The lids of the Schott bottles were loosely left on the containers in order to prevent the loss of distilled water during boiling while still allowing pressure alleviation. The PVA solutions were allowed to cool in ambient room temperature conditions and were then autoclaved at 121 °C for 15 minutes.

The *R. palustris* cells used for immobilization were obtained from a dense liquid culture ($OD_{660\ nm}$ = 4.998). 50 ml centrifuge tubes were filled with the liquid culture and centrifuged for 20 minutes at 3000 RCF. The supernatant was removed and the centrifuge tubes were weighed to get the net cell pellet weight. The cell pellet was then resuspended in a negligible volume of equal parts distilled water and glycerol. This suspension was added to the sterile PVA solution.

The PVA solutions (containing cells) were placed in a hot water bath set at 40 °C. This temperature was chosen so as to keep the solution as fluid as possible without killing the cells. Cryogel beads were formed by dripping the warm PVA solution into liquid nitrogen with the aid of a peristaltic pump. The finished beads were then placed in a sterile container where they were left to thaw at room temperature. The beads were then washed overnight in excess phosphate buffered saline (PBS). The washing procedure was repeated so as to remove any excess glycerol as well as to remove any cells which were not immobilized properly.

5. RESULTS AND DISCUSSION

5.1. Wastewater survey

5.1.1. Summary of wastewater survey

Table 4 is a summary of the key literature findings with respect to the main industrial wastewater streams in South Africa. Table 4 contains the industry, an approximation of the wastewater volumes produced (either as yearly flowrates or as specific effluent flowrates) and the principle wastewater components in each effluent stream were identified. The list of wastewater components for each industry is not exhaustive and in many instances there was substantial doubt as to the respective concentrations, stream composition as well as the variability within a specific effluent stream over time. Nevertheless, many important and difficult to treat effluent components were recognized. Further, Table 4 identifies current treatment methods most commonly employed in industry, and an estimation of the relative pollutant loads, which contextualises this research in the current wastewater treatment field. Finally, wastewater treatment by photosynthetic bacteria, either key single component or whole effluent streams, have been identified from literature. This gives an estimation of the possibility for treatment of each waste stream using the process proposed in this research. The final two columns give an evaluation of the photo-fermentative treatment potential as well as hydrogen production potential for each industry's waste streams. The assessment of photo-fermentative treatment suitability was made by analyzing the wastewater components. Those containing organic material such as sugars, organic acids, carbohydrates, starches and proteins were deemed to have good photo-fermentative treatment potential whereas those containing substances considered harmful to *R. palustris* such as heavy metals, caustic detergents, strong acids and alkalis and inorganic compounds were deemed to have poor photo-fermentative treatment potential. The assessment of hydrogen production potential was also made by analyzing the wastewater components. Those containing nitrogenous compounds such as ammonia were deemed to have poor hydrogen production potential whereas those devoid of nitrogenous compounds were deemed to have good hydrogen production potential. The evaluations were made based on the above-mentioned information and provide a good-medium-poor indication.

Table 4 is neither exhaustive, nor extremely confident in its estimations. However, it provides a tool for identifying potentially photo-fermentatively treatable wastewaters, in a South African context.

Table 4: South African industry wastewaters, with their estimated volumes, principle components, current treatment methodologies and pollutant loads. Additionally, literature citations of purple non-sulphur bacteria metabolizing key components or whole wastewaters and good-medium-poor estimations of photo-fermentative treatment potential and hydrogen production potential.

Industry	WW volumes	Principle WW components	Current treatment methods (if any)	Estimated pollutant loads	WW treatment by photosynthetic bacteria (component or whole stream)	Photo-fermentative treatment potential	Hydrogen production potential
Malt brewing	Micro breweries: 70 - 220 Litres/brew Medium breweries: 7200 - 16200 Litres/brew Beer production: (micro breweries): 17 602 000 L/annum (medium breweries): 351 000 000 L/annum	Organic material Sugars Carbohydrates Starches Proteins Enzymes Aromatic compounds Alcohol	Anaerobic WW treatment process Balancing tank (for pH)	Small breweries: pH: 5.5 COD: 8049 mg/L SS: 554 mg/L TDS: 255 mg/L Total nitrogen: 48 mg/L Total phosphorous: 30 mg/L TOC: 1295 mg/L Soluble orthophosphate: 15 mg/L Nitrates: 0.1 mg/L	Sugars – *R. sphaeroides* (Tao 2008, Kim 2004, Keskin 2012) *R. palustris* (Pott 2013)	Good	Poor
Metal finishing	Specific water index range: 4.23 - 399.57 L/m^2 Specific water index average: 95.02 L/m^2	Immiscible organics: Non-halogenated (oils, greases, solvents) and halogenated (oils, degreasing solvents, paint solvents) Soluble organics: Wetting agents, brighteners, organic ions and ligands	Biological treatment for organics Precipitation Filtration pH treatment Physical separation (flotation or liquid/liquid phase separation) UV irradiation Oxidation (hydrogen peroxide addition)	High pH and low pH High TDS High Cr(vi) High conductivity	None specifically	Medium (organics may be digestible, however high organic content may inhibit growth)	Poor

		Acids and alkalis Particulate material: Metal hydroxides, carbonates, powders, dusts, film residues, metallic particles Metals Complexing agents: Sequestering and chelating agents Nitrogenous materials: Ammonia, Nitrites Cyanides Sulphide Fluorides Phosphated compounds Other salts	Electrochemical recovery or ion exchange				
Soft drink	Effluent Discharge (kL/y): 1000 - 400 000 Specific effluent volume (L/L): 0.1 - 3.8	Wasted soft drink Sugar Syrup Wash water Caustic / detergents and machine lubricant	pH adjustment Holding tank Screening Skimming Settling Irrigation	COD (mg/L): 87 - 725 000 TDS (mg/L): 10 -19 000 SS (mg/L): 55 - 3500 pH: 2.8 - 12.2	Sugars – *R. sphaeroides* (Tao 2008, Kim 2004, Keskin 2012)	Good	Medium

		Nitrates Phosphates Sodium Potassium	High COD streams can be disposed in an anaerobic digester		*R. palustris* (Pott 2013)		
Sorghum beer	Effluent discharge (kL/y): 56 000 - 204 400 (range) Effluent discharge (kL/y): 483 700 (annual/mean) Specific effluent volume – (L/L): 1.54 - 2.92 Specific effluent volume – (L/L): 2,26 (annual/mean)	Organic material Sugars Carbohydrates Starches Proteins Enzymes	Discharge to municipal sewer system	Chemical oxygen demand (mg/L): 2022 COD pollution load (kg/month): 25 010 Specific pollution load – COD (kg/m^3): 3.01 Total dissolved solids (mg/L): 1239 TDS pollution load (kg/month): 15 332 Specific pollution load –TDS (kg/m^3): 1.85 Settleable solids (mg/L): 15 SS pollution load (kg/month): 189.40 Specific pollution load –SS (kg/m^3): 0.02 pH: 3.86	Sugars – *R. sphaeroides* (Tao 2008, Kim 2004, Keskin 2012) *R. palustris* (Pott 2013)	Good	Good
Edible oil	Seed (crude - solvent extraction) SEV: 0.3 m^3/MT oil Seed (refined - physical refining) SEV: 0.1 m^3/m^3 oil Seed (refined - neutralisation) SEV: 0.07 m^3/MT oil	Wastewater from distilling (physical refining), or oil washing and deodorising (chemical refining): free fatty acids, residual gums, pigmented and non-pigmented aromatics, pesticides,	Acid WW: Lime and calcium chloride added to increase pH and as coagulation agent, solids are removed by flocculation and sedimentation Edible oil WW: Gravity separation	Seed oil: pH: 7.2 COD: 33.2 gO2/L FOG/SOG: 41 738 mg/L TP: 1290 mg/L Soya and sunflower oil: pH: 10.7 COD: 6.2 gO2/L FOG/SOG: 5450 mg/L	*R. sphaeroides* COD reduction olive WW - Tao 2008 *R. sphaeroides* (COD reduction olive WW) - Karapinar 2006	Good	Good

	Seed (refined - soap splitting) SEV: 0.04 m^3/MT oil Seed (condensate from neutralisation) SEV: 0.055 m^3/MT oil Seed (refined - chemical refining) SEV: 0.15 m^3/MT oil Olive oil (traditional pressing): 2 - 5 m^3/t oil Olive oil (3-phase extraction): 6 - 8 m^3/t oil Olive oil (2-phase extraction): 0.33 - 0.35 m^3/t oil	degumming acids (phosphoric acid, citric acid), residual soaps The acid wastewater generated from soap splitting (chemical refining only): sulphuric acid, free fatty acids, other organics Final wastewater: emulsifying agents, metal catalysts (nickel or palladium)	Skimming Dissolved air filtration Chemical coagulation Flocculation Electrocoagulation Biological treatment Olive oil WW Irrigation	TP: 88 mg/L (orthophosphate) SO42-: 274 g/L TSS: 811 g/L Na: 174 mg/L Acid wastewater: pH: 2.8 COD: 4.4 gO2/L FOG/SOG: 1600 mg/L TP: 401 mg/L (orthophosphate) SO42-: 3978 g/L TSS: 1577 g/L Na: 833 mg/L Olive oil (traditional pressing) BOD5: 2.2 - 6.2 x 10^4 mg/L Olive oil (3-phase extraction) BOD5: 1.3 - 1.4 x 10^4 mg/L Olive oil (2-phase extraction) BOD5: 9 -102 x 10^4 mg/L Olive oil (traditional pressing: COD: 5.9 - 16.2 x 10^4 mg/L Olive oil (3-phase extraction) COD: 3.9 - 7.8 x 10^4 mg/L	*R. palustris* (COD reduction olive WW) - Pintucci 2014 *R. sphaeroides* (COD reduction olive WW) - Eroglu 2004		

				Olive oil (2-phase extraction) COD: 12 - 13 x 10^4 mg/L Olive oil (traditional pressing) TSS: 6.5 x 10^4 mg/L Olive oil (3-phase extraction) TSS: 6.5 x 10^4 mg/L Olive oil (2-phase extraction) TSS: 12 x 10^4 mg/L Olive oil (traditional pressing) pH: 4.6 - 4.9 Olive oil (3-phase extraction) pH: 5.2 Olive oil (2-phase extraction) pH: 4.5 - 5			
Red meat abattoir	7.2 m^3/annum	Organic matter (excretion) Blood (COD: 400 000 mg/L) Fat	Anaerobic digestion system	COD: 730 - 8900 mg/L SS: 1700 - 3048 mg/L TDS: 595 - 2805 mg/L Total Kjeldahl Nitrogen: 1 - 24 mg/L	None specifically	Good	Medium (nitrogen may inhibit H_2)
Laundry	200 - 400 m^3/day	Heavy metals Organic solvents Alkalis/builders Antichlors Bleaches Softeners Sours	Alkaline wastewaters treated with acidifying chemicals Coagulation and flocculation chemicals	SS: >1000 mg/L COD: up to 20 000 mg/L BOD: >1300 mg/L FOG: >1100 mg/L TP: 0.5 - 3.8 mg/L as P TKN: 3.2 - 80 mg/L as N pH: 8.5 - 10.6	None specifically	Medium (low digestible organic content)	Medium

		Starches Variety of detergents Phosphates Salts Soil FOG's Residual textile material Microbes Adsorbable organically-bound halogens Sodium Nitrogen Ammonia Boron Surfactants Each one of above has many components: Dodecylbenzene sulfonic acid Sodium salt Poly(oxy-1,2-ethanediyl),alpha-(4-nonylphenyl)-omega-hydroxy-,branched Dimethyloctadecel benzyl ammonium chloride Cocamidopropyl Betaine Sodium Hydroxide EDTA Sodium Hydroxide Alkyl dimethyl benzyl ammonium chloride	for the settling of colloids Cross-flow microfiltration Dissolved air flotation (DAF) Ultrafiltration (UF) Coagulation and flocculation (removes SS and colloids) Activated carbon filtration (removes organic pollutants) Oxidation (ozone, UV, chlorination and peroxides) Biological methods (degrades organic material and nutrients) Ion exchange technology (reduce water hardness) Membrane filtration (removes small	Absorbance (600 nm): 0.059 - 0.236 A lot of variability (see individual laundry processes)			

		7-dietylamonio-4-methylcoumarin Amylase Sodium Metasilicate Benzyl Acetate Polyethylene Glycol Butylated hydroxytoluene N,N-Dimethyldodecylamine Oxide Sodium Hypochlorite Sodium Bisulfate Sodium Thiosulfate Phosphoric Acid Dimethyloctadecyl benzyl ammonium chloride Starch	particles and colloidal material) Electro-chemical methods (e.g. electro-coagulation and electro-flotation)				
Tanning and leather finishing	50 m^3 / 1000 kg raw hides	Sulphates Sulphides Chlorides Chromium Acid salts Salts Organic waste Inorganics (salts) Ammonium ions Dyestuffs Organic solvents Heavy metals Biocides, surfactants, degreasers, Enzymes	Pretreatment: Mechanical screens Grit removal apparatus Distribution wells Equalisation tanks Primary treatment: Physicochemical processes Physical separation (screening) Fat traps Chemical coagulants and flocculants Settling tank for pH correction	COD: 242 kg/1000 kg raw hides/skin BOD: 100 kg/1000 kg raw hides/skin SS: 150 kg/1000 kg raw hides/skin Chromium: 5 kg/1000 kg raw hides/skin Sulphide: 10 kg/ 1000 kg raw hides/skin Chloride: 10 kg/1000 kg raw hides/skin pH: 3.46 - 12.7 COD: 412 - 12 091 mg/L TDS: 812 - 22 244 mg/L	Dye decolourisation *R. palustris* (Reactive black dye) (Wang 2008) Organic acids Many examples – see Appendix A	Medium	Medium

		Lime, sodium sulphide, sodium hydrosulphide, caustic soda Ammonium sulphate, ammonium chloride, formic acid, proteolytic enzymes Salt, sulphuric acid, formic acid, sodium formate, fungicide Trivalent chrome tanning salts (wet blue) or glutaraldehyde (wet white) Magnesium oxide and sodium bicarbonate Surfactants Sodium bicarbonate, sodium formate Syntans, resins, vegetable tannins Dyestuffs	Chrome and sulphides precipitation Sedimentation Clarifier Secondary treatment: Biological treatment (aerobic or anaerobic) Conventional activated sludge system Anaerobic ponds	TSS: 839 - 1268 mg/L Sulphates: 702 - 2780 mg/L Chlorides: 474 - 12 022 mg/L Total chromium: 1.31 - 46 mg/L			

		Fatliquors (emulsified oils) Formic acid Salt, manure, blood, dirt, globular skin proteins, biocides, surfactants and degreasers Decomposed hair keratin, globular skin proteins, saponified fractions of natural skin fat, sulphides, lime and caustic soda Hide fats and fleshings in the form of a solid waste Calcium salts, sulphide residues, organic acids, degraded proteins, residual proteolytic enzymes, ammonium sulphate and ammonium chloride Salt, trivalent chrome (wet blue), glutaraldehyde (wet white), magnesium and sodium salts, sulphuric acid and formic acid					

		Surfactants Sodium salts Residues of retanning chemicals, dyestuffs and fatliquors					
Paper and pulp	SEV: 0.08 - 84.5 m^3/t Effluent discharge: 45 - 92 260 m^3/d	Lignin, organic components from wood, chlorine dioxide, caustic soda, hydrogen peroxide	Clarification Activated sludge Dissolved air flotation Belt press Irrigation Anaerobic digestion	COD: 165 - 3853 mg/L SS: 34 - 2260 mg/L Conductivity: 211 - 4970 uS/cm pH: 6.5 - 8.5 N: 0.1 - 1.2 mg/L P: 0.1 - 0.5 mg/L SO4: 16 - 565 mg/L Cl: 11 - 340 mg/L Na: 10 - 740 mg/L	*R. palustris* (Degradation of 2-Chlorophenol in effluents from paper and wood industry) - Mutharasaiah 2012	Medium (undetermined digestibility)	Good
Iron and steel	SEV: 0.9 - 3.6 m^3/t	Heavy metals Hydrocarbons Cyanide compounds Nitrogen compounds Oil/grease	Clarification Activated sludge Dissolved air flotation Evaporation Ammonia stripper	COD: 13 mg/L Conductivity: 81 - 3493 uS/cm Fe: 0.02 - 3.28 mg/L Cr: 0.01 - 0.14 mg/L Cl: 2.5 - 145 mg/L Na: 0.12 - 160 mg/L Mn: 0.03 - 1.72 mg/L pH: 8.8 - 10.2	None specifically	Poor (low organic content)	Medium
Cane sugar processing	SEV: 0.1 - 11.53 kL/ton cane	Inorganics Sugars Sugar metabolites Phosphates	Physiochemical processes (settling and flocculation) Coagulation with alum in the presence of lime Biological oxidation	pH: 4.2 - 10.1 COD: 110 – 6820 mg/L BOD5:COD: 0.08 - 0.6 TSS: 5 - 1480 mg/L TDS: 1008 - 1480 mg/L TP: 0.8 - 5.9 mg/L	Sugar wastewaters *R. sphaeroides* Karapinar 2006	Good	Good

			Solids settling Biological treatment (pond systems, activated sludge system) Natural percolation through sand Dilution	Cl: 50 - 522 mg/L	Kapdan 2006 Keskin 2012 Yetis 2000 *R. palustris* Fascetti 1998 Singh 1993 Xu 2013		
Textile	80 - 90 % of specific water intake 12 128 - 284 075 kL/year (dyers and finishers) 29 - 75 500 kL/year (producers) 106 800 - 840 000 kL/year (technical and traditional textiles)	Acids Alkalis Bleach Dyes Salts Surfactants Stabilisers Starch Reactive dyes Vat dyes Sulphur dyes Direct dyes Disperse Dyes Indigo dyes (being phased out) Chromium, copper, zinc, lead or nickel (some effluents) Pesticides, microorganisms or contaminants (some effluents) Detergents	Primary: Screening Sedimentation Homogenisation Neutralisation Mechanical/chemical flocculation Secondary: Aerobic and anaerobic treatment Aerated dams Activated sludge Trickling filters Oxidation ditches and ponds Tertiary: Membrane techniques Adsorption Oxidation Electrolytic precipitation	COD: 291 - 1831 mg/L NH3+: 0.03 - 31.45 mg/L PO4-: 1.71 - 31.29 mg/L pH: 6.62 - 10.10 Conductivity: 78.52 - 1159.45 mS/m TSS: 53.00 - 746.00 mg/L TS: 753.33 - 7256.00 mg/L Nitrate: 0.45 - 4.09 mg/L Chlorides: 66.36 - 3140.45 mg/L Sulphates: 64.24 - 308.63 mg/L	*R. palustris* (Reactive black 5 decolourization of textile effluent) - Wang 2008	Medium	Medium

		Natural oils Waxes Carboxymethyl cellulose Polyvinyl alcohol Chlorine-based bleaches (organic halogens as trichloromethane) Caustic soda Halogens Metals Amines Salts Reducing/oxidizing agents Antifoaming agents Volatile organic compounds Organic solvents Inorganic salts	Electro-chemical processes Ion exchange Photocatalytic degradation Thermal evaporation				
Poultry	56 - 10 944 000 kL/annum	High BOD Salts Nutrients Bacterial contamination	Anaerobic or settling ponds Facultative ponds Mechanically forced aerated ponds Naturally aerated ponds Dissolved air flotation (DAF) cells Septic tanks Screening Holding tank Flocculation pH adjustment	COD: 1000 - 6000 mg/L SS: 51 - 1500 mg/L EC: 75 - 2000 mS/m TDS: 500 - 4000 mg/L TKN: 21 - 250 mg/L NH4-N: 0 - 100 mg/L TP: 5 - 50 mg/L PO4-P: 0 - 250 mg/L Na: 0 -500 mg/L Cl: 101 - 500 mg/L	None specifically	Good	Poor (high nitrogen content)

			Segregation of concentrated process streams Anaerobic biogas system Anaerobic digestion				

Wastewaters selected for growth experiments are based on the photo-fermentative treatment potential classification listed in Table 5, where those classified as poor are rejected and those classified as medium or good were selected for further evaluation. The wastewater components listed in Table 5 are extracted from Table 4.

Table 5: Summary of wastewater components found in the effluents from various industries and assessment of their photo-fermentative treatment potential.

Industry	WW components	Photo-fermentative treatment potential
Malt brewing	Organic material, sugars, carbohydrates, starches, proteins, enzymes, aromatic compounds, alcohol	Good
Metal finishing	Immiscible organics, soluble organics, acids and alkalis, metals, complexing agents, nitrogenous materials, cyanides, fluorides, sulphide, phosphate compounds and salts	Medium
Soft drink	Wasted soft drink, sugar, syrup, wash water, caustic / detergents and machine lubricant, nitrates, phosphates, sodium, potassium	Good
Sorghum beer	Organic material, sugars, carbohydrates, starches, proteins, enzymes	Good
Edible oil	Free fatty acids, residual gums, aromatics, pesticides, residual soaps, emulsifying agents, metal catalysts and sulphuric acid	Good
Red meat abattoir	Organic matter (excretion), blood (COD: 400 000 mg/L), fat	Good
Laundry	Heavy metals, organic solvents, bleaches, alkalis/builders, antichlors, softeners, sours, starches, detergents, phosphates, salts, soil, nitrogen, ammonia, surfactants, boron, sodium, fats, oils and grease	Medium
Tanning and leather finishing	Sulphates, sulphides, chlorides, chromium, acid salts, salts, organic waste, inorganics, ammonium ions, dyestuffs, organic solvents and heavy metals	Medium
Paper and pulp	Lignin, organic components from wood, chlorine dioxide, caustic soda, hydrogen peroxide	Medium
Iron and steel	Heavy metals, hydrocarbons, cyanide compounds, nitrogen compounds, oil/grease	Poor
Cane sugar processing	Inorganics, sugars, sugar metabolites, phosphates	Good
Textile	Acids, alkalis, bleach, dyes, salts, surfactants, stabilizers, starch, reactive dyes, vat dyes, sulphur dyes, direct dyes, disperse dyes, chromium, copper, zinc, lead and nickel	Medium
Poultry	High BOD, salts, nutrients, bacterial contamination	Good

5.2. Chemical oxygen demand

The initial COD content and nitrogen content of the various collected wastewaters can be seen in Table 6. The wastewaters listed in Table 6 differ to those identified in Table 5. This was due to the availability of the industrial wastewaters. A number of companies from various industries were contacted and the wastewater streams available were obtained. The pollutant loads, in the form of COD and nitrogen, contained in the wastewaters listed in Table 6 clearly demonstrates the need for the treatment of these effluents before they can be safely disposed to municipal wastewater treatment plants or be used for irrigation or process water purposes. The initial COD content of the wastewater samples given in Table 6 were determined according to the method given is section 4.4.2.3.

Table 6: Initial COD content of the various collected wastewaters.

Number	Wastewater	Initial COD (mg/L)	Initial Nitrogen (mg/L)
1	Winery processing	5035	12
2	AD effluent	32950	50
3	Brewery	20200	48
4	Vinasse	90 000	2400
5	Olive processing	3570	20
6	Tannery	2200	Not available
7	Fish processing	3094	Not available
8	Paper and pulp	1562	Not available
9	Dye	Not applicable	Not applicable

5.3. Growth experiment and COD reduction

For each wastewater, a run was conducted with 100% wastewater as well as a 50% dilution with media. The media contained no carbon and no nitrogen source. For cases where no growth was observed, higher dilution rates were used (10 % wastewater in media). Reasons for no growth are attributed to a component or components in the wastewater being too concentrated and therefore toxic and inhibitory to the growth of *R. palustris*. Further, the wastewater could also be lacking in an essential nutrient or component which is necessary for the survival of *R. palustris*. Experiments were conducted at 35 °C. In order to suitably choose reactor conditions for optimal wastewater treatment, a fundamental study of the effects of temperature on *R. palustris* growth was required. Current literature has not investigated this thoroughly. Research has been conducted by a PhD candidate at Stellenbosch University on the growth of *R. palustris* under various temperature conditions, when grown on glycerol and glutamate as test carbon and nitrogen sources, respectively, in the temperature controlled photobioreactor set-up described in section 4.3. Temperatures were varied between 30 °C and 45 °C. Analysis of this research showed that as the temperature increased, the growth rate also

increased given by greater cell dry weights. The implication is that higher reactor temperatures will result in faster wastewater purification. With previous studies largely conducted at 30 °C, a reaction temperature of 35 °C was selected in order to achieve improved wastewater treatment. Additionally, industrial photobioreactors are likely exposed to direct sunlight which will tend to raise the reaction temperature above 30 °C.

If growth is identical at all dilution rates, then there is no limiting or inhibitory compound within the wastewater. If growth is better at higher dilution levels compared to the pure wastewater, then the wastewater contains a component at a concentration which is either killing or inhibiting the growth of *R. palustris*.

Secondly, based on hydrogen gas production rates and yields, these experiments also aim to answer whether the particular wastewater is conducive to hydrogen production. If negligible or no hydrogen is produced, the wastewater contains an already fixed source of nitrogen, for example ammonia or the ratio of nitrogen to carbon is too high.

The experiments listed in section 5.3. were batch experiments with free cells.

For cases when wastewater is diluted with media, the COD was calculated as according to equation 11. Sample calculations for these procedures can be seen in Appendix G. The COD of media was determined to be 640 mg/L according to the method given in section 4.4.2.3. and is assumed to be constant.

$$COD_{measured} = \varphi_{media}(COD_{media}) + \varphi_{wastewater}(COD_{wastewater}) \qquad [11]$$

5.3.1. Winery wastewater

Growth curves of *R. palustris* grown in winery wastewater can be seen in Figure 8. Growth was observed for both the diluted and undiluted cases. For diluted winery wastewater the biomass concentration increased by an average of 68 % and for undiluted winery wastewater the biomass concentration increased by an average of 74 %. Sample calculations for these procedures can be seen in Appendix G. The average maximum biomass concentration for diluted winery wastewater and for undiluted winery wastewater was 0.35 g cell dry weight/L and 0.43 g cell dry weight/L respectively. Analysis of Figure 8 shows that undiluted winery wastewater showed improved growth of *R. palustris* when compared to diluted winery wastewater. This is an extremely positive and promising result as it means the winery wastewater is not deficient of nitrogen and other trace minerals which are essential for the biological system. Further, if the batch system is scaled up for high volume winery wastewater

treatment or if a continuous mode of treatment is employed, large savings in operating costs may be achieved as no additional media will need to be added to the system.

Winery wastewater contains sugars, organic acids and aldehydes which have been shown, in isolation, to be ideal substrates for growth by photo-fermentative bacteria such as *R. palustris.* Therefore, the composition of the winery wastewater explains the observed growth of *R. palustris*.

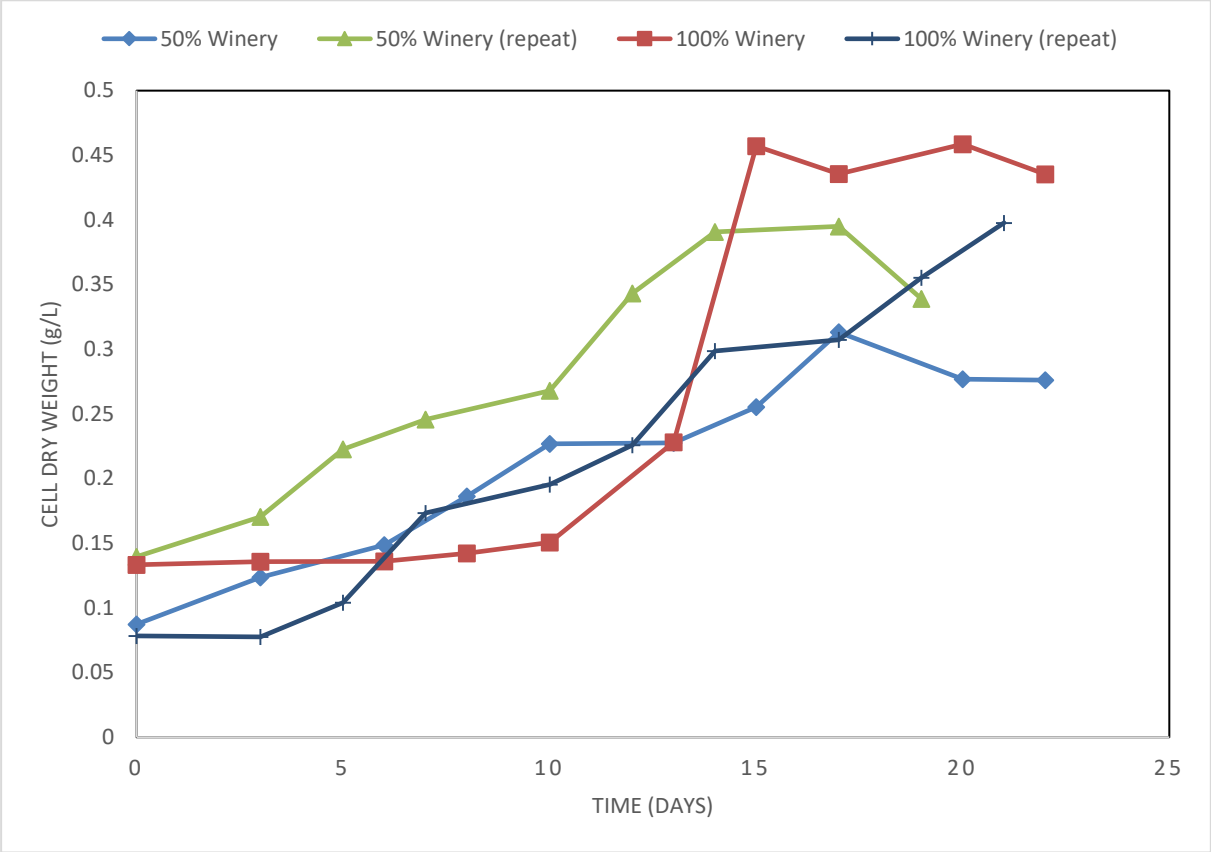

Figure *8: The growth of R. palustris on undiluted winery wastewater (□, +) and winery wastewater diluted by 50 % with carbon and nitrogen free growth media (◊, Δ).*

Figure 9 shows the COD depletion of diluted and undiluted winery wastewater over time. The COD decreased for both the diluted and undiluted cases. For diluted winery wastewater the COD decreased by an average of 90 % and for undiluted winery wastewater the COD decreased by an average of 72 %. Sample calculations for these procedures can be seen in Appendix G. The COD decrease is a result of *R. palustris* metabolizing and consuming the organic components in the wastewater.

The maximum specific COD consumption rate for diluted winery wastewater was 5.18 g COD/g biomass and occurred after 9 days. The maximum specific COD consumption rate for undiluted winery wastewater was 5.04 g COD/g biomass and occurred after 11 days. Despite observing a COD reduction for both diluted and undiluted winery wastewater, the time taken to reach the maximum specific COD consumption rate and therefore the overall COD reduction was lengthy and commonly longer than current alternative wastewater treatment technologies (Oller, Malato and Sánchez-Pérez, 2011).

However, the COD reduction rate can be increased through various methods. These include but are not limited to increasing the biomass concentration as well as the illumination.

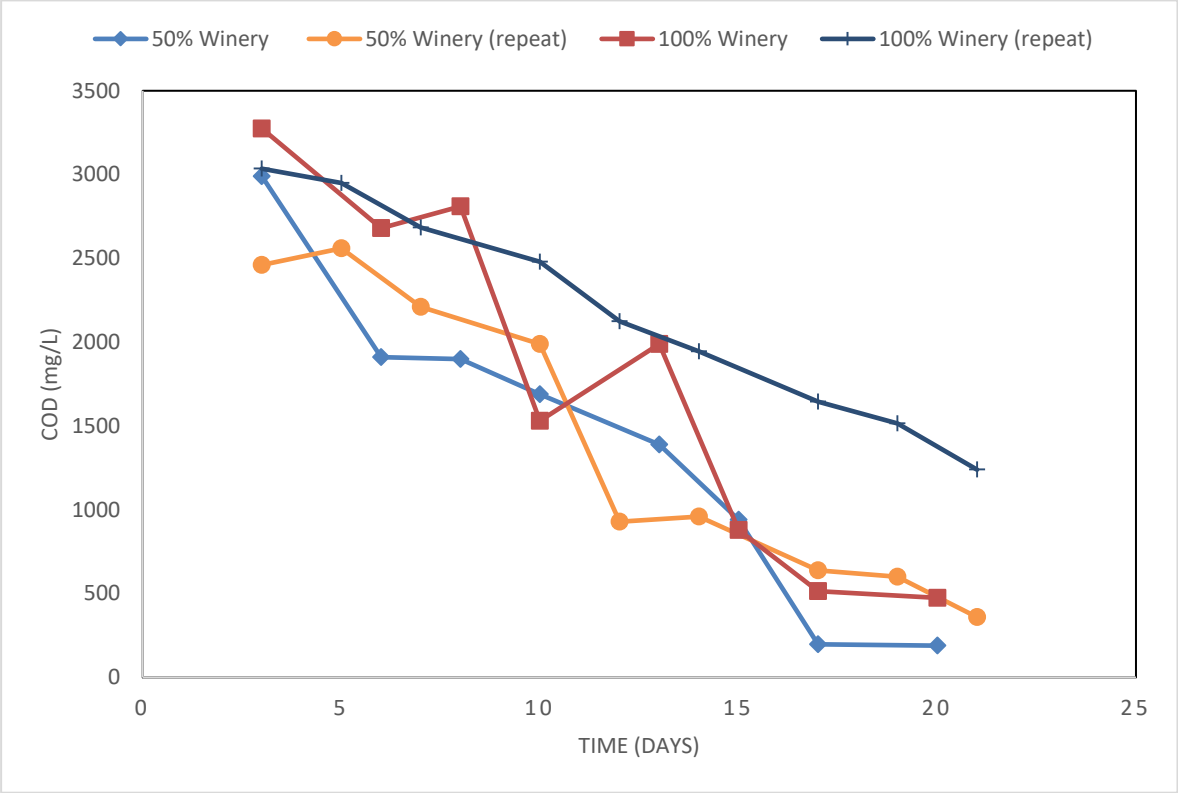

Figure *9: COD depletion of winery wastewater versus culture age.*

5.3.2. Anaerobic digester effluent

Experiments were conducted with undiluted anaerobic digester effluent as well with anaerobic digester effluent diluted by 50 % with carbon and nitrogen free growth media. No growth was observed in these experiments and the *R. palustris* cells died. Therefore, the dilution was increased to 90 % where growth was observed. The aim of this research was not to determine the point at which bacterial growth first occurred when increasing wastewater dilution, but rather to generate a fuller picture of the bacterial growth achieved under certain conditions on the wastewaters. For this reason, large dilution rate increments were employed to the wastewaters when no growth was observed. Additionally, given the number of wastewaters tested, and the time taken for experiments to run, large dilution rate increments were necessary to generate sufficient results within a reasonable time span. Growth curves of *R. palustris* grown in anaerobic digester effluent can be seen in Figure 10. For diluted anaerobic digester effluent the biomass concentration increased by an average of 81 %. The average maximum biomass concentration for diluted anaerobic digester effluent was 0.62 g cell dry weight/L. For both of the AD effluent experimental runs, the growth curve reaches a stationary phase at approximately 7 days, where the bacteria multiply at the same rate at which they die. This is possibly

due to the depletion of an essential nutrient which is required for growth. The number of cells is now limited by a growth factor. Based off initial batch wastewater treatment tests on the various wastewaters, an experiment duration of 21 days was selected. This time scale was chosen as it was observed that this allowed sufficient time for the COD reduction and bacterial growth to stabilise. In other words, no further COD reduction and no increase in biomass was observed after 21 days. Further, certain experiments were shortened when an earlier COD reduction and bacterial growth stabilization were observed. This is the case for experiments on AD effluent, where the tests were terminated after 17 days.

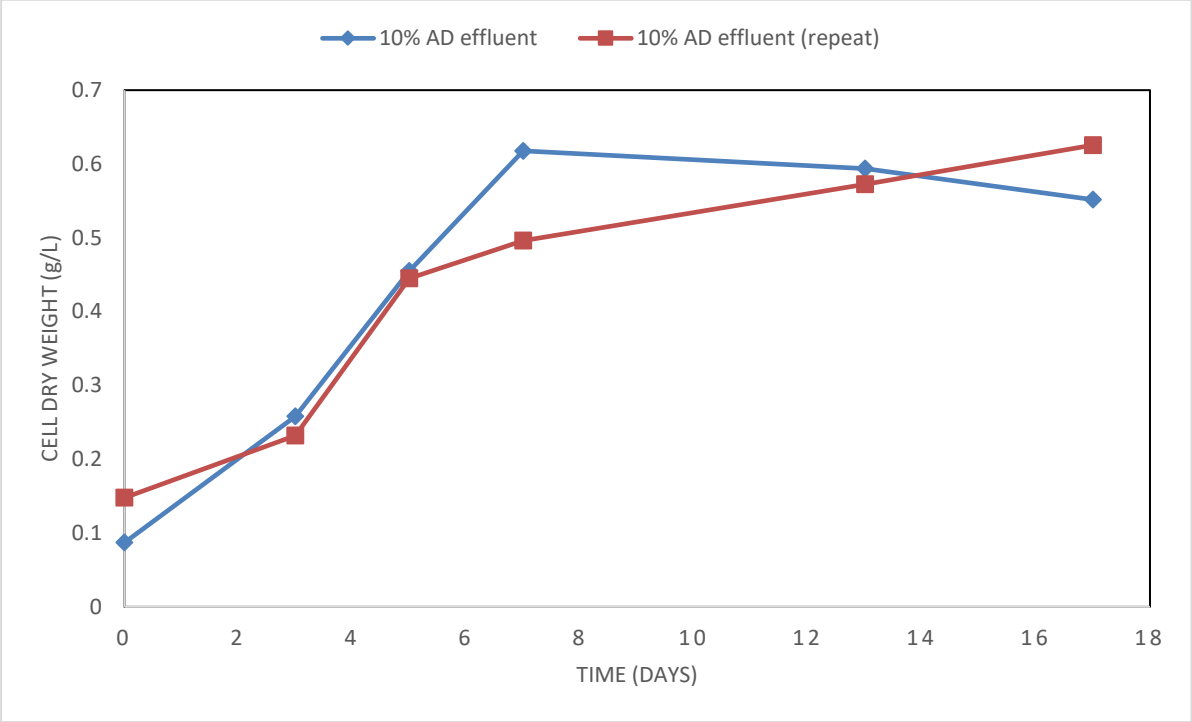

Figure *10: The growth of R. palustris on anaerobic digester effluent diluted by 90 % with carbon and nitrogen free growth media (◊, □).*

Figure 11 shows the COD depletion of diluted anaerobic digester effluent over time. The COD decreased from an average of 10040 mg/L to 4115 mg/L on day 17. For the diluted anaerobic digester effluent, the COD decreased by an average of 59 %. The rate of COD depletion is unaffected by the decrease in microbial growth after day 7. The first data point in Figure 11 is for day three. This was due to wastewater being inoculated on a Friday, the experiment left to run over the weekend, and the first sample being analysed on the following Monday.

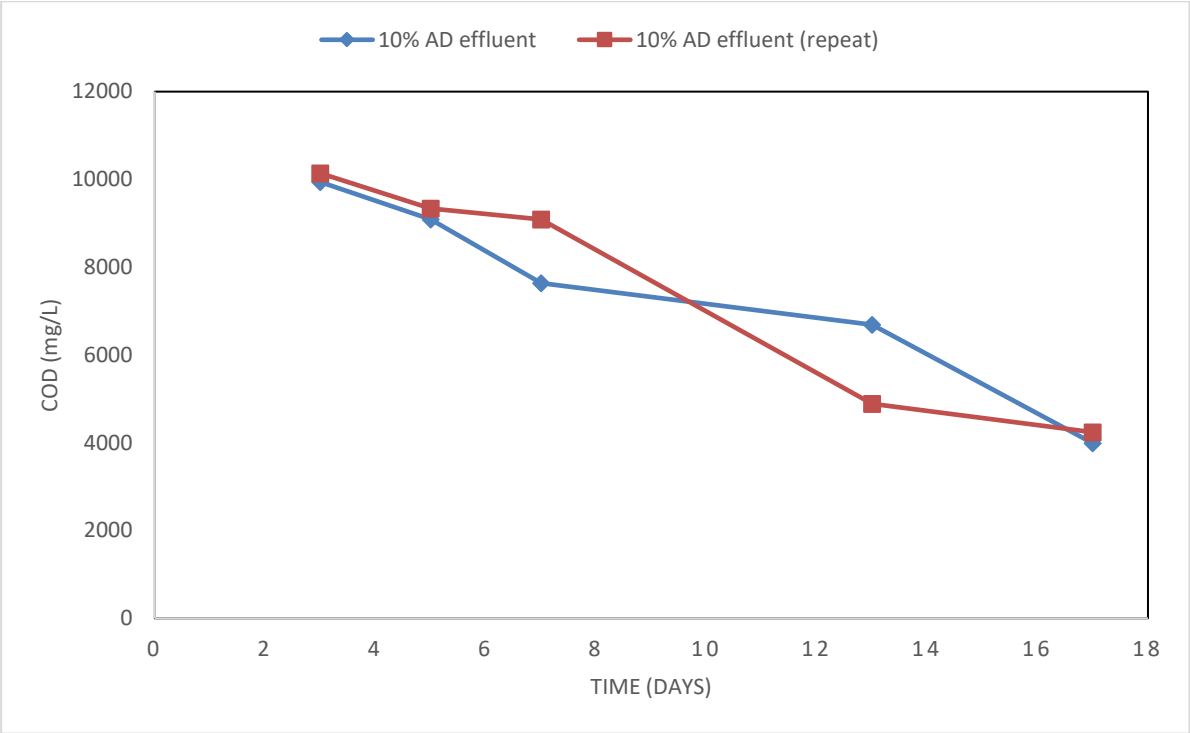

Figure *11: COD depletion of anaerobic digester effluent versus culture age.*

5.3.3. Brewery wastewater

Experiments were conducted with undiluted brewery wastewater as well with brewery wastewater diluted by 50 % with carbon and nitrogen free growth media. No growth was observed in these experiments and the *R. palustris* cells died. Therefore, the dilution was increased to 90 % where growth was observed. The metabolism of purple non-sulphur bacteria generally becomes inactive at pH values less than 5 which results in no cell growth (Tao *et al.,* 2008). The pH of 50 % diluted brewery wastewater was 4 whereas the pH of 90 % diluted brewery wastewater was 6. Therefore, dilution of brewery wastewater is required in order for cell growth to occur.

Growth curves of *R. palustris* grown in brewery wastewater can be seen in Figure 12. For diluted brewery wastewater the biomass concentration increased by an average of 78 %. The average maximum biomass concentration for diluted brewery wastewater was 0.38 g cell dry weight/L. Brewery wastewater contains sugars, carbohydrates and starches which are good carbon sources for photo-fermentation.

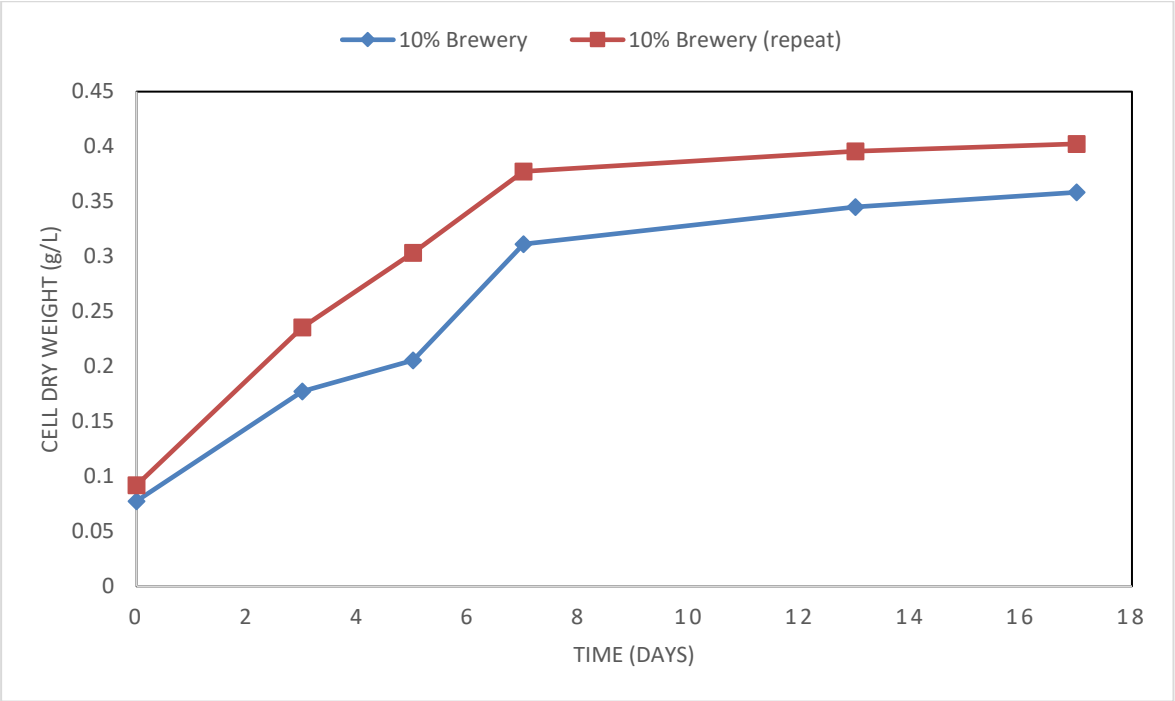

Figure *12: The growth of R. palustris on brewery wastewater diluted by 90 % with carbon and nitrogen free growth media (◊, □).*

The BOD and COD of untreated brewery wastewater was 13742 mg/L and 20200 mg/L respectively. This results in a BOD/COD ratio of 0.68 which means the brewery wastewater is considered to be easily treatable by biological means and contains highly biodegradable components. Figure 13 shows the COD depletion of diluted brewery wastewater over time. The COD decreased from an average of 6565 mg/L to 290 mg/L on day 17. For the diluted brewery wastewater, the COD decreased by an average of 96 %. The maximum specific COD consumption rate for diluted brewery wastewater was 7.73 g COD/g biomass and occurred after 10 days.

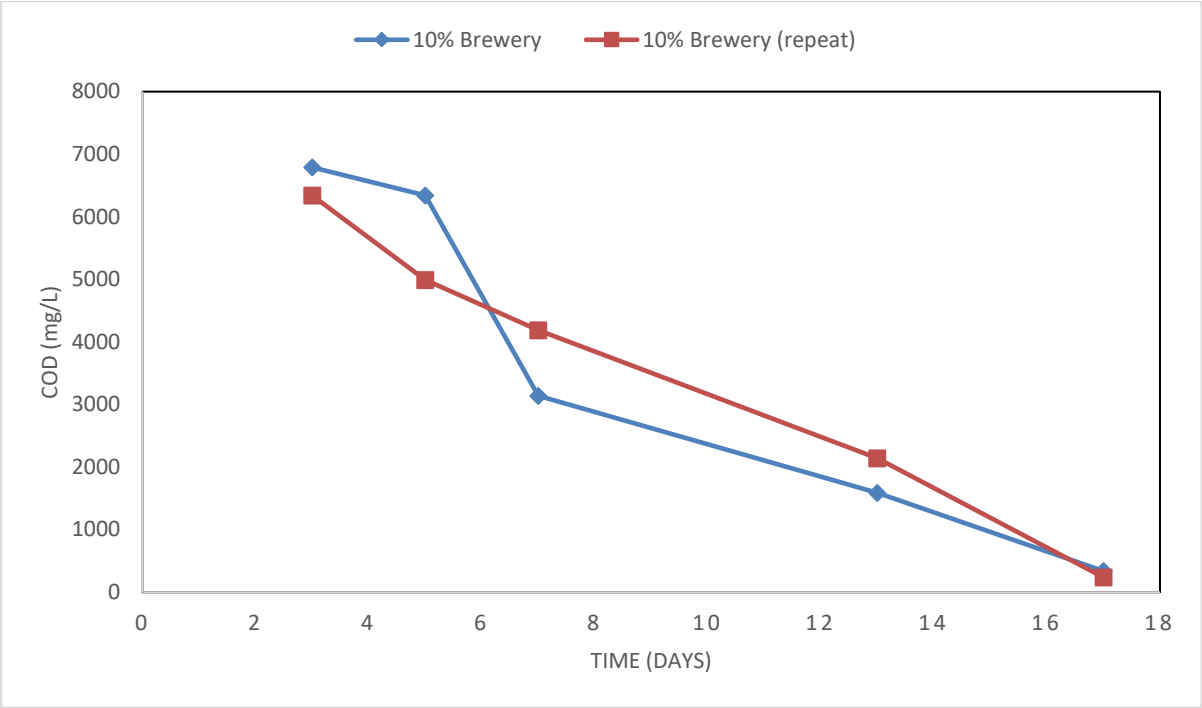

Figure *13: COD depletion of brewery wastewater versus culture age.*

5.3.4. Vinasse waste

Experiments were conducted with undiluted vinasse waste as well with vinasse waste diluted by 50 % and 90 % with carbon and nitrogen free growth media. No growth was observed in these experiments and the *R. palustris* cells died. Therefore, the dilution was increased to 99 % where growth was observed. A dilution of 99 % is not realistic. Challenges at this dilution rate are that very small volumes of vinasse waste can be treated as the reactor volume almost entirely consists of media. Additionally, the operating expenses of this wastewater treatment will be extremely costly as large volumes of media will need to be produced. This also adds further complications to the downstream processes as media separation and recovery steps will need to be implemented so that media can be recycled. Growth curves of *R. palustris* grown in vinasse waste can be seen in Figure 14. For diluted vinasse waste the biomass concentration increased by an average of 56 %. The average maximum biomass concentration for diluted vinasse waste was 0.15 g cell dry weight/L. Vinasse contains acetic acid, phenolic compounds and carbohydrates which are good substrates for growth. However, vinasse is also highly acidic (pH 3 – 5) and contains high concentrations of sulphur and salts which resulted in the poor growth (Mshoperi, 2018). Further, even at 99 % dilution, the liquid remained extremely dark black and non-transparent. Therefore, light attenuation for this wastewater was extremely high which resulted in the cells receiving very little energy in the form of light.

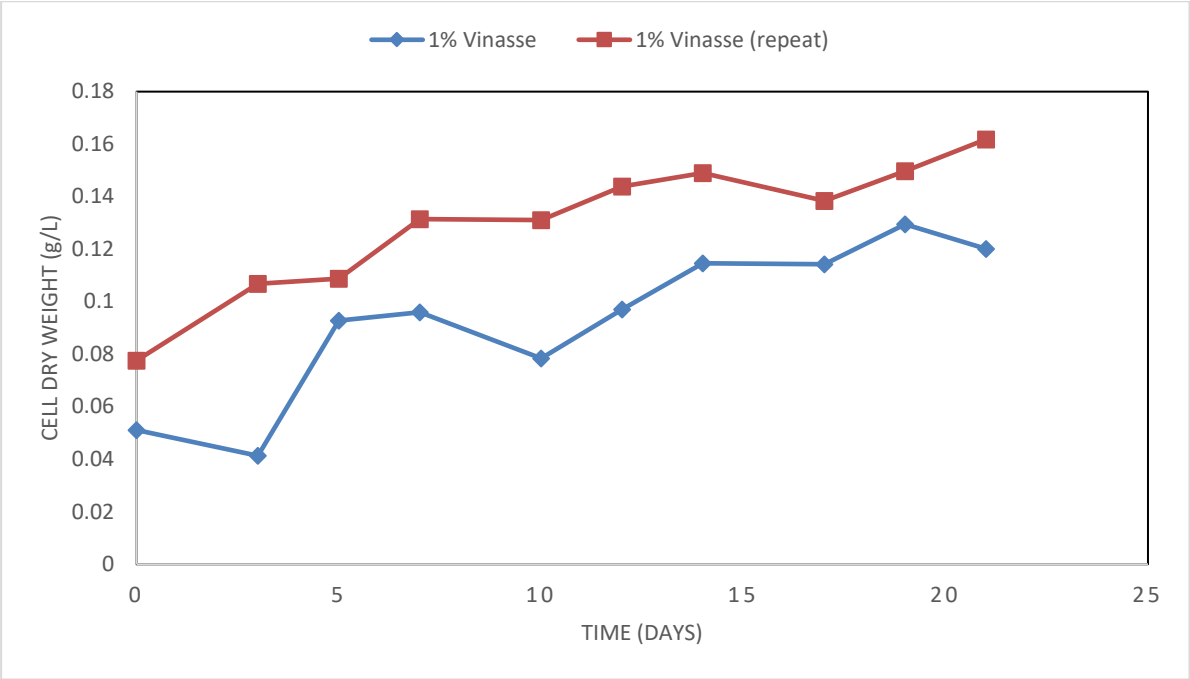

Figure *14: The growth of R. palustris on vinasse waste diluted by 99 % with carbon and nitrogen free growth media (◊, □).*

Figure 15 shows the COD depletion of diluted vinasse waste over time. The COD decreased from an average of 74140 mg/L to 29640 mg/L on day 21. For the diluted vinasse waste, the COD decreased by an average of 59 %. Despite poor growth of *R. palustris* in vinasse waste, a substantial COD decrease was observed. While conditions were not suitable for growth, there was an abundance of organic material metabolized by *R. palustris*. After day 12 the COD content of the vinasse waste remained relatively constant. Vinasse has a BOD of 43000 mg/L and a COD of 90000 mg/L resulting in a BOD/COD ratio of 0.48. This means vinasse waste is on the threshold to be considered easily treatable by biological means and may contain some toxic components. It also explains the halt in COD reduction after day 12.

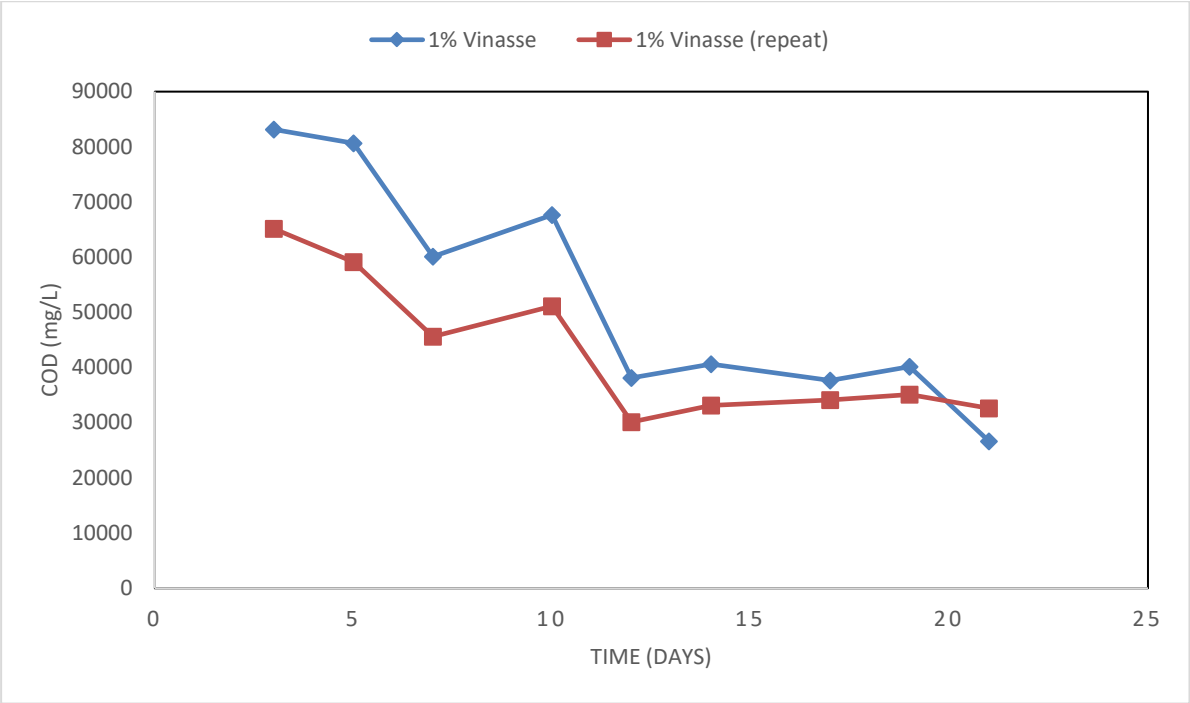

Figure *15: COD depletion of vinasse waste versus culture age.*

5.3.5. Olive processing wastewater

Growth curves of *R. palustris* grown in olive processing wastewater can be seen in Figure 16. Growth was observed for both the diluted and undiluted cases. For diluted olive processing wastewater the biomass concentration increased by an average of 42 % and for undiluted olive processing wastewater the biomass concentration increased by an average of 27 %. The average maximum biomass concentration for diluted olive processing wastewater and for undiluted olive processing wastewater was 0.31 g cell dry weight/L and 0.18 g cell dry weight/L, respectively. Analysis of Figure 16 shows that diluted olive processing wastewater showed improved growth of *R. palustris* when compared to undiluted olive processing wastewater.

Due to the lack of an accelerated or exponential growth phase, it can be suggested that the olive processing wastewater is deficient of nutrients required for growth. This is highlighted by the slightly improved growth observed for the diluted case where bulk nutrients, trace elements, thiamine and vitamin B12 have been added. It can also be concluded that the wastewater is free of any toxic compounds which kill *R. palustris* cells, as a stable cell population is present in the reactor over the 21 day period. Growth is also not inhibited by pH, as the pH of untreated olive processing wastewater is 5.8 (Appendix E). Therefore, the bacterial growth in this wastewater is growth rate limited and the cells are not killed by a toxic component or a component which is present in a very high concentration.

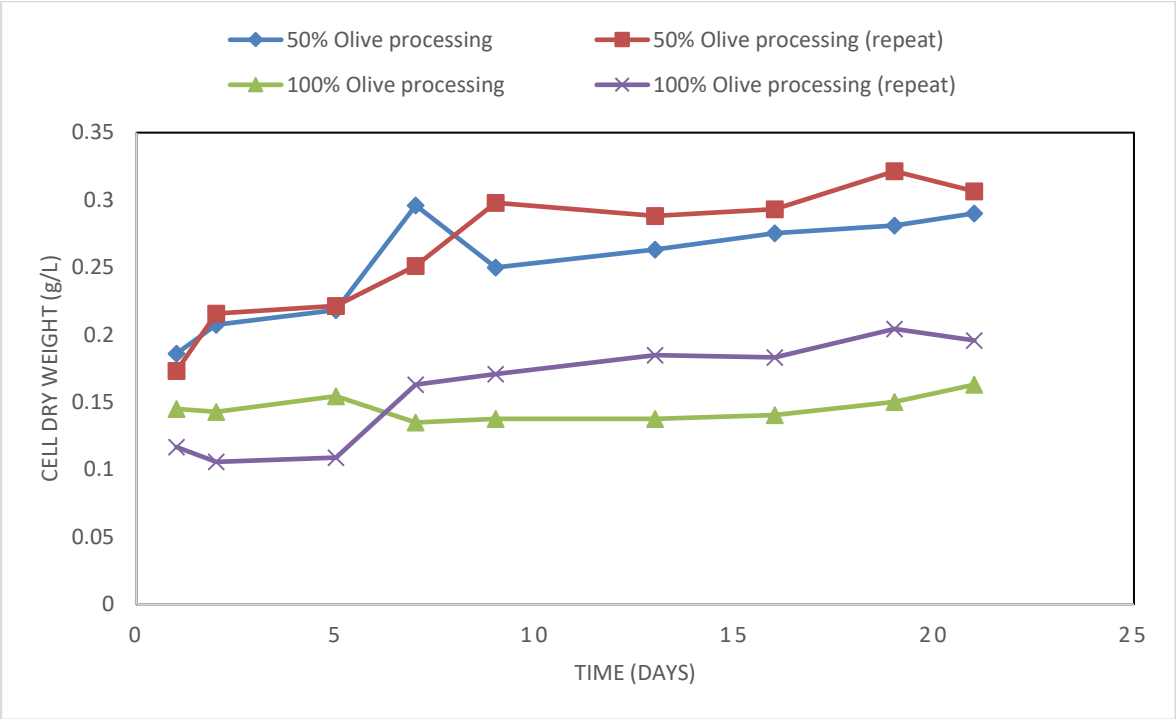

Figure *16: The growth of R. palustris on undiluted olive processing wastewater (Δ, x) and olive processing wastewater diluted by 50 % with carbon and nitrogen free growth media (◊, □).*

Figure 17 shows the COD depletion of diluted and undiluted olive processing wastewater over time. A significantly greater COD decrease was observed for the diluted olive processing wastewater when compared to the undiluted olive processing wastewater. For diluted olive processing wastewater the COD decreased by an average of 79 % and for undiluted olive processing wastewater the COD decreased by an average of 28 %. For the undiluted olive processing wastewater there is a clear difference in the COD removal between the two runs. The COD does not decrease in the first experimental run but does decrease in the repeat run. The reason that the COD does not decrease in the first experimental run is due to the lack of bacterial growth in the first experimental run. This can be seen in Figure 16 where the biomass remains relatively constant in the first experimental run but increases in the second experimental run. The increased COD removal for the diluted olive processing wastewater compared to the undiluted case is attributed to the improved growth which is seen in the diluted case. Olive processing wastewater contains fatty acids which can easily be biodegraded but also contains aromatics which are more difficult to biodegrade through photo-fermentation by *R. palustris.*

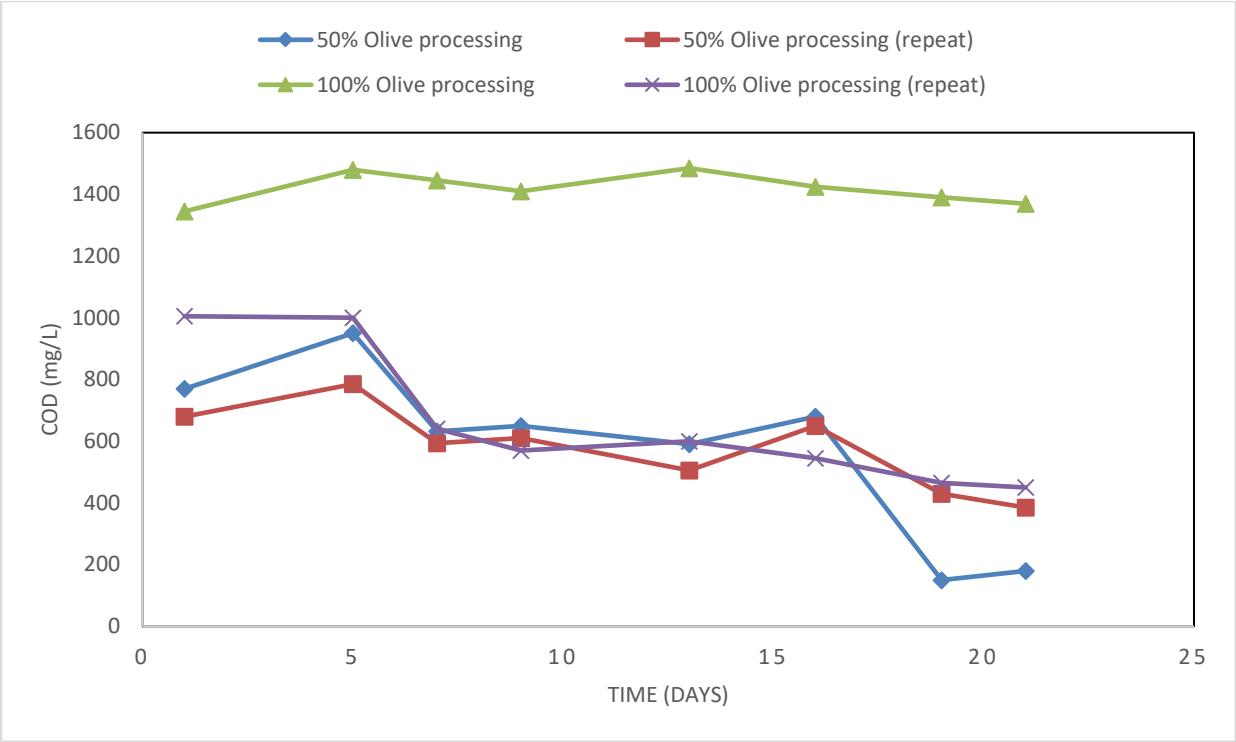

Figure *17: COD depletion of olive processing wastewater versus culture age.*

5.3.6. Tanning and leather finishing wastewater

Growth curves of *R. palustris* grown in tanning and leather finishing wastewater can be seen in Figure 18. Growth was observed for both the diluted and undiluted cases. For diluted tanning and leather finishing wastewater the biomass concentration increased by an average of 46 % and for undiluted tanning and leather finishing wastewater the biomass concentration increased by an average of 20 %. The average maximum biomass concentration for diluted tanning and leather finishing wastewater and for undiluted tanning and leather finishing wastewater was 0.44 g cell dry weight/L and 0.25 g cell dry weight/L respectively. Analysis of Figure 18 shows that diluted tanning and leather finishing wastewater showed improved growth of *R. palustris* when compared to undiluted tanning and leather finishing wastewater.

The growth observed in the tannery wastewater follows similar trends to that of olive processing wastewater and appears to be limited by a growth factor. In other words, an essential nutrient required for growth has been depleted.

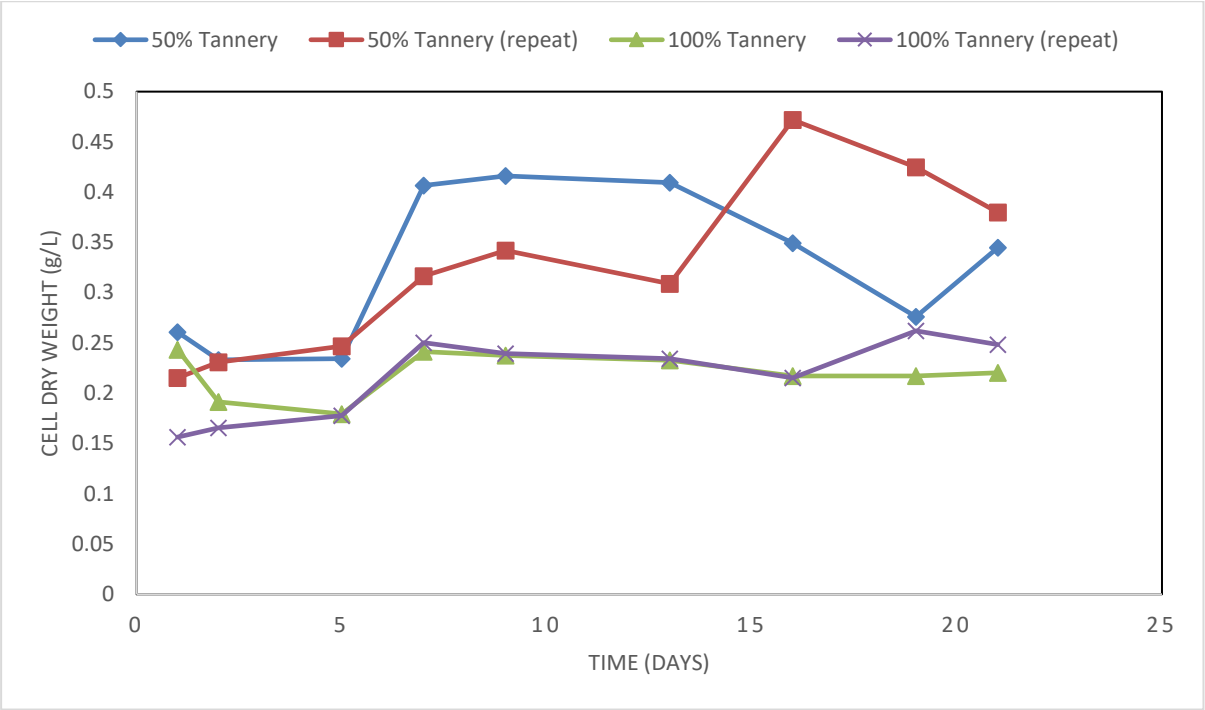

Figure *18: The growth of R. palustris on undiluted tanning and leather finishing wastewater (Δ, x) and tanning and leather finishing wastewater diluted by 50 % with carbon and nitrogen free growth media (◊, □).*

Figure 19 shows the COD depletion of diluted and undiluted tanning and leather finishing wastewater over time. A significantly greater COD decrease was observed for the diluted tanning and leather finishing wastewater when compared to the undiluted tanning and leather finishing wastewater. For diluted tanning and leather processing wastewater the COD decreased by an average of 86 % and for undiluted tanning and leather finishing wastewater the COD decreased by an average of 27 %.

The increased COD removal for the diluted tannery wastewater compared to the undiluted case is attributed to the improved growth which is seen in the diluted case. Tannery wastewater contains a large variety of undesired components for photo-fermentation. These include inorganic substances such as sulphates, sulphides, chlorides and high concentrations of salts. The pH of tannery wastewater can also vary widely, ranging between 3.5 – 12.7 (Table 4 in section 5.1.) which is another hurdle for optimal photo-fermentative conditions.

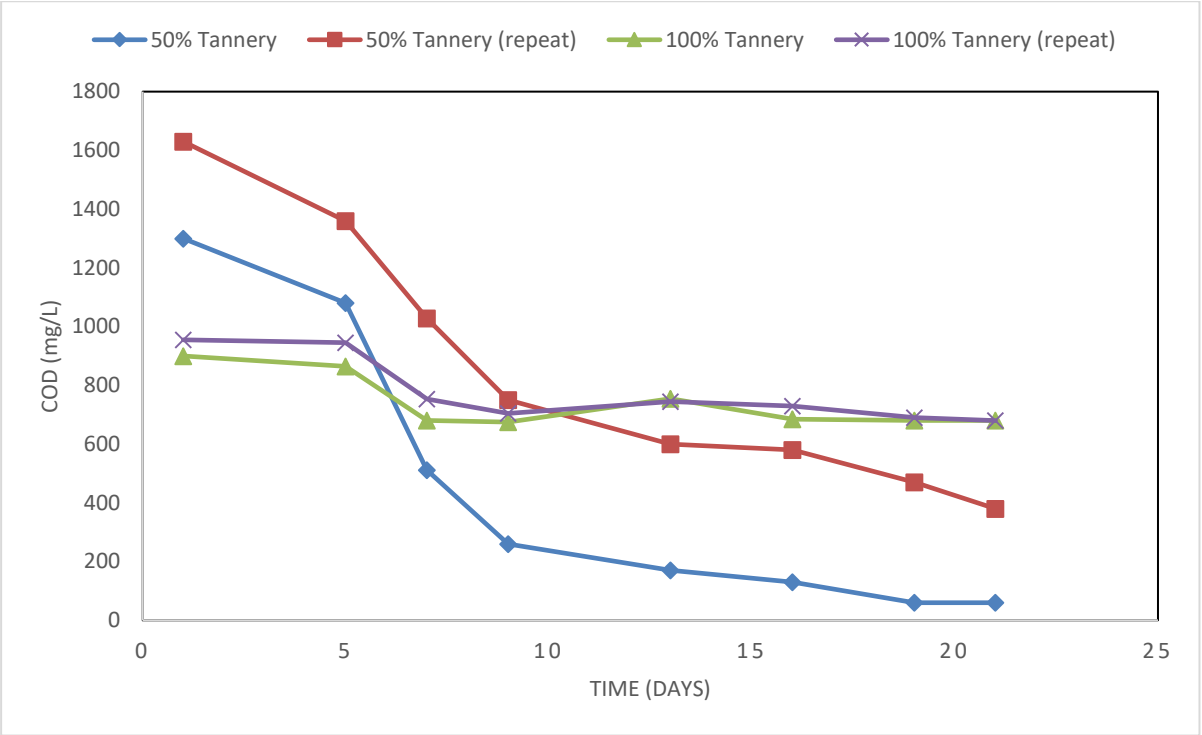

Figure *19: COD depletion of tanning and leather finishing wastewater versus culture age.*

5.3.7. Fish processing wastewater

Growth curves of *R. palustris* grown in fish processing wastewater can be seen in Figure 20. Growth was observed for both the diluted and undiluted cases. For diluted fish processing wastewater the biomass concentration increased by an average of 20 % and for undiluted fish processing wastewater the biomass concentration increased by an average of 23 %. The average maximum biomass concentration for diluted fish processing wastewater and for undiluted fish processing wastewater was 0.45 g cell dry weight/L and 0.34 g cell dry weight/L, respectively. Analysis of Figure 20 shows that growth was poor for both diluted and undiluted fish processing wastewater.

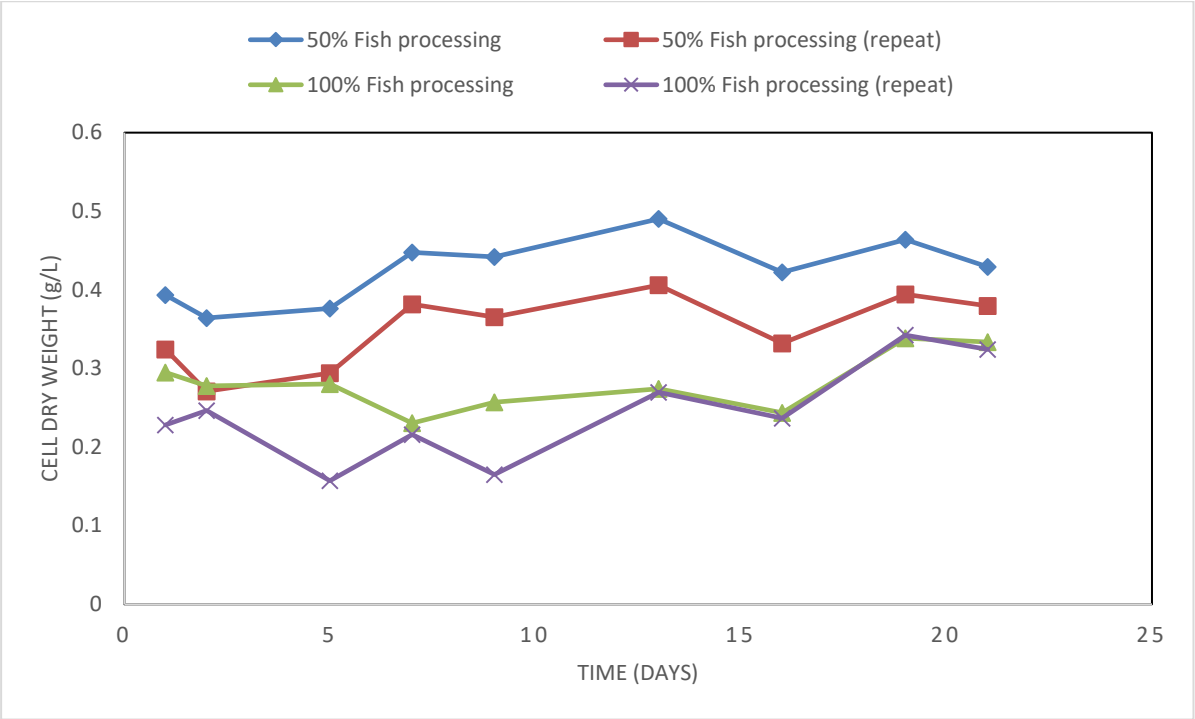

Figure *20: The growth of R. palustris on undiluted fish processing wastewater (Δ, x) and fish processing wastewater diluted by 50 % with carbon and nitrogen free growth media (◊, □).*

Figure 21 shows the COD depletion of diluted and undiluted fish processing wastewater over time. A significantly greater COD decrease was observed for the diluted fish processing wastewater when compared to the undiluted fish processing wastewater. For diluted fish processing wastewater the COD decreased by an average of 83 % and for undiluted fish processing wastewater the COD decreased by an average of 25 %.

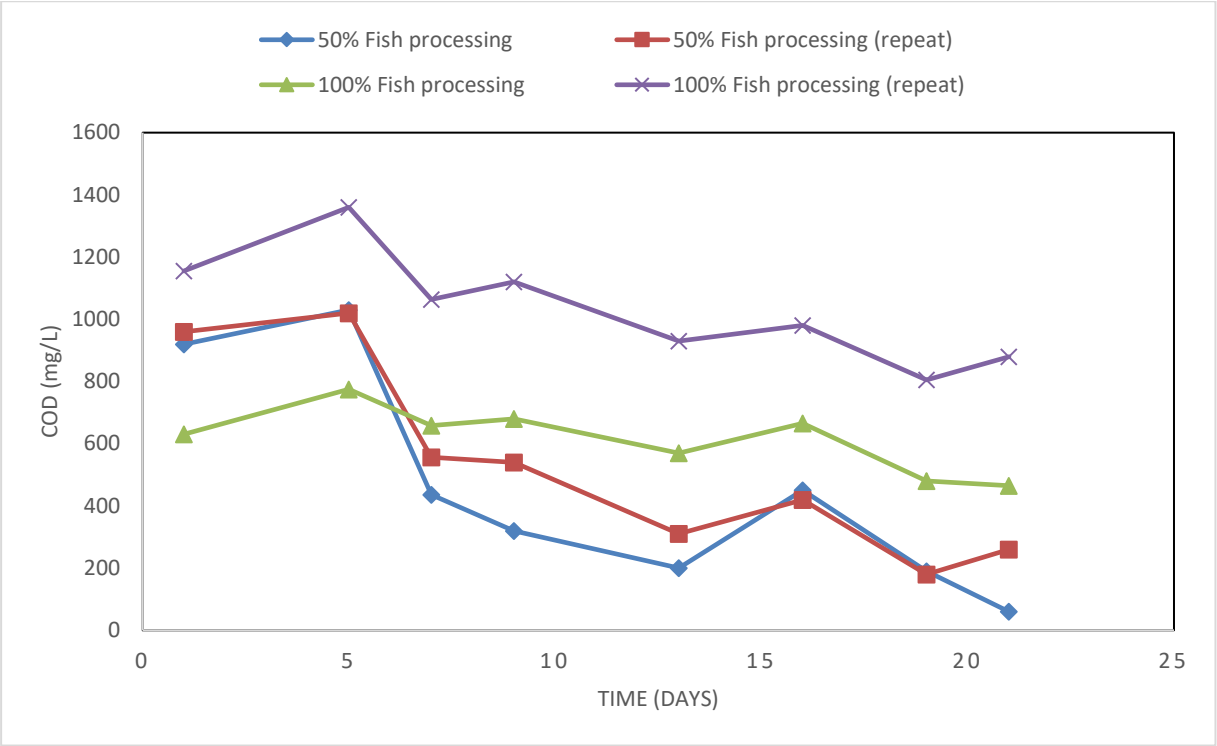

Figure *21: COD depletion of fish processing wastewater versus culture age.*

5.3.8. Paper and pulp wastewater

Growth curves of *R. palustris* grown in paper and pulp processing wastewater can be seen in Figure 22. Growth was poor for both the diluted and undiluted cases with negligible growth observed after day 9. Further, for three out of the four experiments, the biomass concentration began decreasing after day 13. This indicates the presence of a toxic compound which results in the death of the cells. The average maximum biomass concentration for both diluted and undiluted paper and pulp processing wastewater was 0.09 g cell dry weight/L. Paper and pulp processing wastewater contains bleaching chemicals such as chlorine dioxide, hydrogen peroxide as well as caustic soda which explains the poor growth of *R. palustris* in this effluent.

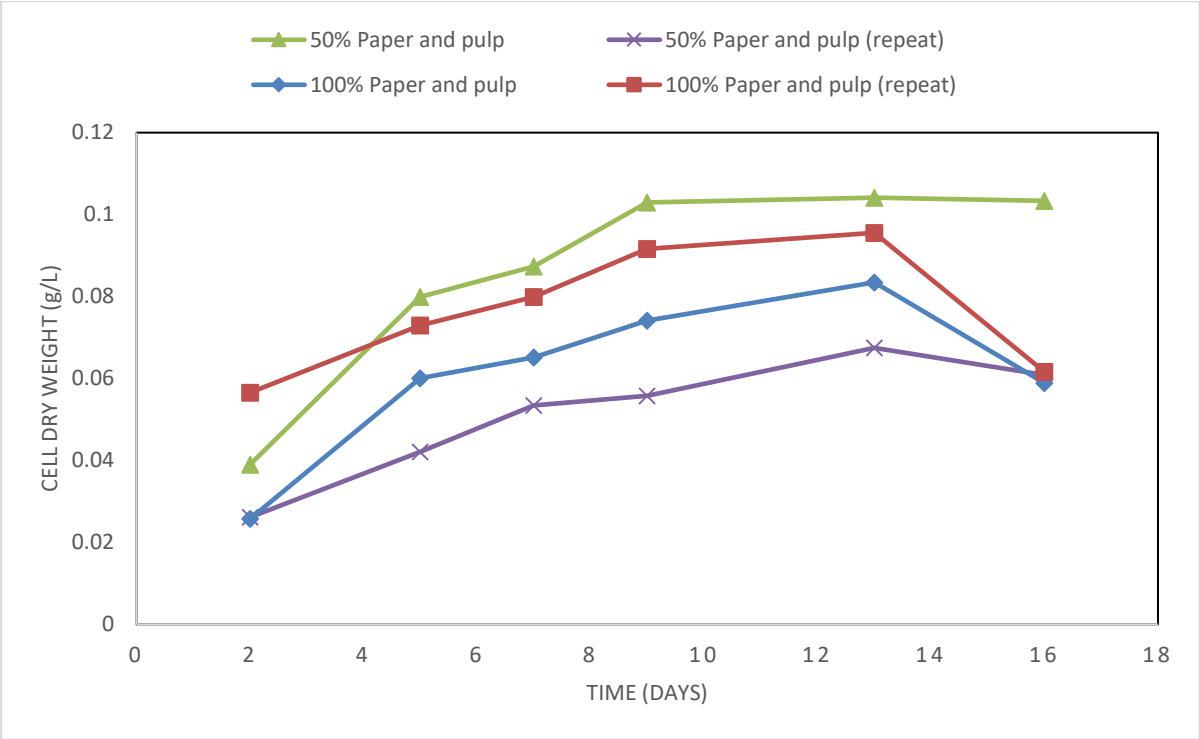

Figure *22: The growth of R. palustris on undiluted paper and pulp processing wastewater (◊, □) and paper and pulp processing wastewater diluted by 50 % with carbon and nitrogen free growth media (Δ, x).*

Figure 23 shows the COD depletion of diluted and undiluted paper and pulp processing wastewater over time. The COD content for both the diluted and undiluted cases stopped decreasing at day 9. Either a toxic component in the effluent killed the *R. palustris* cells, or the cells completely metabolized the biodegradable organic portion of the wastewater and were left with no usable carbon substrate.

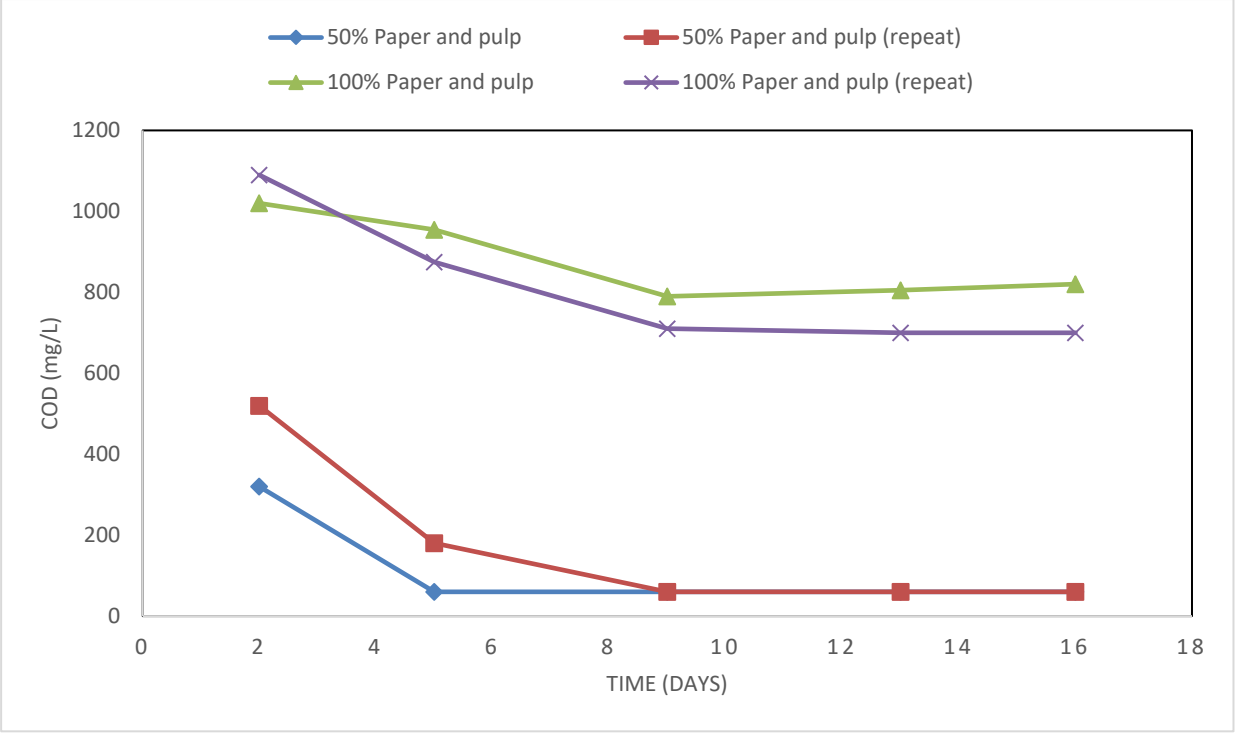

Figure *23: COD depletion of paper and pulp processing wastewater versus culture age.*

5.3.9. Textile dye wastewater

Experiments were conducted with undiluted dye wastewater as well with dye wastewater diluted by 50 % with carbon and nitrogen free growth media. No growth was observed in these experiments and the *R. palustris* cells died. With dye wastewater differing from other industrial wastewaters in that it mainly contains the dye molecules and salts, and not a range of organic substances and nitrogen. Therefore, experiments were repeated with the addition of glutamate (2M) and glycerol (5M) in an attempt to generate *R. palustris* growth. However, still no growth was observed.

Further, approaches in reducing BOD from the majority of wastewaters is well known. However, dye wastewater has proven more challenging to treat owing to their synthetic nature and complex aromatic molecular structures. These structures are also designed to prevent fading when in contact with light, sweat, water, soaps and other oxidising agents. This results in molecules which are less responsive to biodegradation and which are more stable (Banat *et al.,* 1996).

Therefore, as an alternative to generating growth curves and COD removal curves, the UV-VIS spectra of the dye wastewater was analysed. This was done in order to determine absorbance peaks which are caused by the presence of dye chemicals. The UV-VIS spectra of dye wastewater can be seen in Figure 24. By monitoring the absorbance at specific wavelengths, the decolourization efficiency of the dye wastewater can be determined which is a measurement of colour removal. Analysis of Figure 24 shows that three main peaks are present. These are at wavelengths of 288 nm, 523 nm and 546 nm.

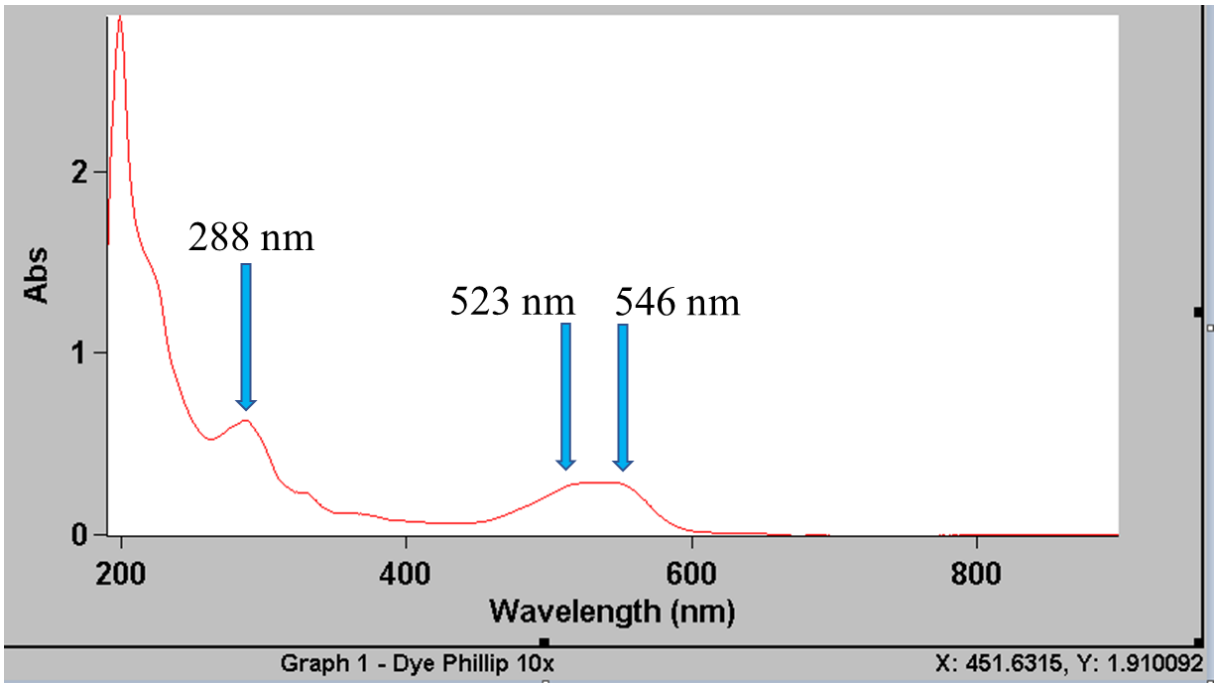

Figure *24: UV-VIS spectra of dye wastewater.*

Despite the obtained dye wastewater sample containing three dyes (Yellow 3RS, Red 3B5 and Navy), it visually appeared to be a dark red colour. Red azo dyes are known to have maximum absorbance wavelengths between 500 nm and 550 nm. Form Figure 24 it can be seen that there is a maximum absorbance interval between 523 nm and 546 nm. The absorbance of the dye wastewater at 288 nm, 523 nm and 546 nm was then monitored over a 23 day period in order to determine if any of the dyes have been biodegraded. Figure 25 shows the absorbance of the dye wastewater at the wavelengths of interest.

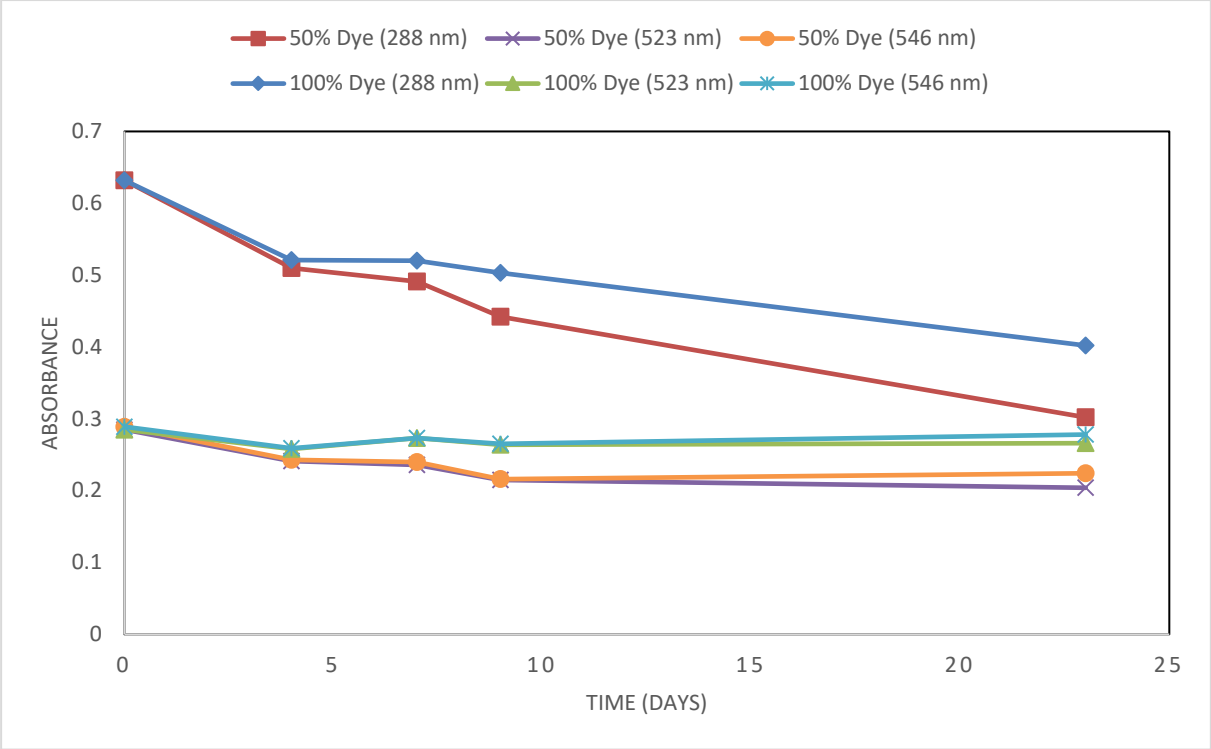

Figure *25: Absorbance of dye wastewater.*

The decolourization efficiency was then calculated according to equation 12, where A_i is the initial absorbance and A_f is the absorbance of the decolourized sample. Sample calculations for these procedures can be seen in Appendix G. The results are shown in Table 7. The decolourization efficiency is substantially greater for the diluted textile dye wastewater when compared to the undiluted textile dye wastewater. This is due to a greater biomass concentration present in the diluted wastewater as a result of the presence of media.

Decolourization efficiency:

$$F = \frac{(A_i - A_f)}{A_i} \times 100 \qquad [12]$$

Table 7: Decolourization efficiency of diluted and undiluted dye wastewater at three wavelengths causing absorbance peaks.

	Decolourization efficiency (%)	
Wavelength (nm)	**50 % Dye**	**100 % dye**
288	52.22	36.39
523	28.42	6.67
546	22.49	3.81

5.3.10. Repeatability

Repeatability is an area of concern with the results presented in section 5.3. Reasons for erratic data arise due to variability in the collected wastewater samples. For some experiments, repeat runs were conducted with wastewater that was frozen and defrosted multiple times whereas for the first experimental run the wastewater was only defrosted once. Therefore, wastewater ageing plays a role in inconsistent data. Additionally, experiments were conducted on wastewater samples collected at different times from the same process streams. Therefore, although the overall process is the same, there are varying components and varying concentrations of the components in the wastewater.

Differences in cell dry weight starting points are attributed to the first wastewater samples being taken on a Monday when the wastewaters were inoculated on a Friday. Therefore, two days are present where the bacteria can grow at different rates between the initial and repeat experiments.

5.3.11. Summary of growth experiments and COD reduction

Figure 26 shows a comparison of the biomass concentration increase for the various wastewaters and Figure 27 shows a comparison of the COD decrease for the various wastewaters. From Figure 26 it can be seen that winery wastewater, AD effluent and brewery wastewater performed well with respect to bacterial growth. All these wastewater exhibited a biomass concentration increase of greater than 60 %. Of these (winery wastewater, AD effluent and brewery wastewater), the winery wastewater and the brewery wastewater showed large COD reductions (> 70 %). Analysing the winery wastewater and the brewery wastewater, it can be seen that the winery wastewater performed well without being diluted or at low dilution rates, whereas the brewery wastewater and to be highly diluted in order to perform well. Therefore, winery wastewater was selected as the wastewater most suitable for treatment by *R. palustris*.

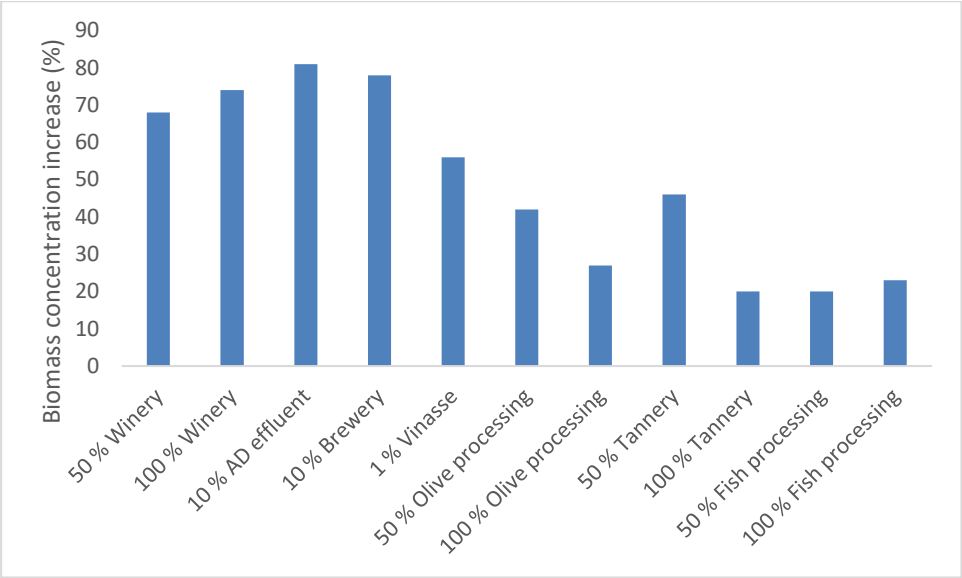

Figure 26: A comparison of the biomass concentration increase for the various wastewaters.

The growth in this study is comparatively low when compared to other studies. For instance, when *R. sphaeroides* was grown on pure components as substrates, the cell concentrations were 1.13 g cdw/L for glucose, 0.74 g cdw/L for glycerol. 0.61 g cdw/L for sucrose, 0.86 g cdw/L for starch, 1.81 g cdw/L for malate, 1.07 g cdw/L for lactate and 0.80 g cdw/L for acetate (Kim *et al.,* 2006). The greatest cell concentration achieved in this study was with diluted AD effluent and was 0.63 g cdw/L. Diluted AD effluent also showed the greatest increase in biomass concentration with an 81 % increase. However, many of the wastewaters showed biomass concentration increases less than 50 %. Essentially, growth of less than 100 % means that *R. palustris* growth is low and that the organism is not well suited for its environment. This is due to the wastewater lacking an essential nutrient required for growth or the presence of toxic components in high concentrations.

When *R. palustris* was grown in sago effluent medium there was a large and significant difference between the biomass concentration when mineral supplementation and carbon source was added to the effluent medium and cases when non mineral supplementation and carbon source was added to the effluent medium. When supplementation was implemented the maximum cell concentration was 0.8 g cdw/L whereas when no supplementation was added the maximum cell concentration was 0.1 g cdw/L (Getha *et al.,* 1998). This shows that effluent stream are lacking in their composition when it comes to supporting bacterial growth.

When *R. sphaeroides* was grown in olive mill wastewater by (Eroglu *et al.,* 2004) extremely high dilutions were required for bacterial growth to occur. At 80 % dilution with distilled water the cell concentration was 0.05 g cdw/L and at 99 % dilution with distilled water the cell concentration was 0.55 g cdw/L. Therefore, work carried out in the study on olive mill wastewater can be considered relatively successful as a cell concentration of 0.32 g cdw/L was achieved at a 50 % dilution.

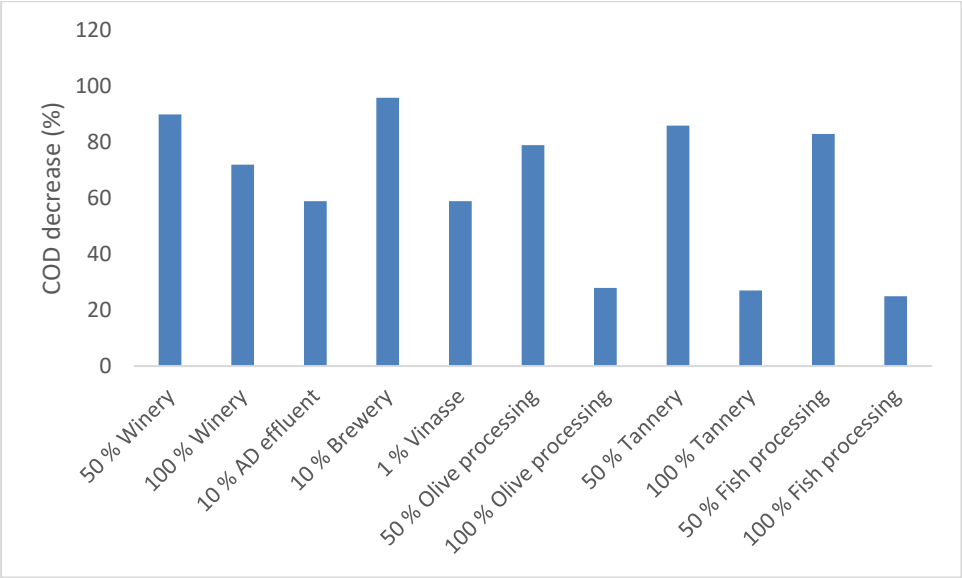

Figure 27: A comparison of the COD decrease for the various wastewaters.

It is important to note that that even though the growth of *R. palustris* was poor in the some of the wastewaters, the COD reduction was still good. This implies that nutrients are missing which are required for growth but that the wastewater is not killing the organism. This is an important conclusion as it means that the wastewaters are applicable for biological wastewater treatment if the missing nutrients are added. Further, it is observed that the rate of COD decrease is not affected by the growth phase of the organism and that the COD starts decreasing as soon as the bacteria start growing. Therefore, if the wastewater treatment process is operated industrially, and the bacteria are kept alive, the COD will decrease without the bacteria need to grow.

5.3.12. Comparison to literature

The trends observed throughout section 5.3. are expected. Firstly, for the vast majority of the wastewaters, the diluted experiments showed improved growth and COD decrease when compared to the undiluted cases. This is due to the diluted cases containing media which contains the essential nutrients and trace elements which *R. palustris* requires for growth.

For wastewaters containing sugars (winery, brewery) a COD reduction ranging between 72 % and 96 % was achieved. This compares well to work done by (Tao *et al*., 2008) who achieved a COD reduction of 88 % with *R. sphaeroides* on sugar containing synthetic medium. For wastewaters containing organic acids (winery, AD effluent and tannery) a COD reduction of ranging between 59 % and 90 % was achieved. This also compares well to work done by (Chen *et al.,* 2008) who achieved a COD reduction of 72 % with *R. palustris (WP3-5)* on organic acid containing synthetic medium. COD decreases have also been reported by (Tao *et al*., 2008), (Karapinar, 2006) and (Eroglu, 2004) all using *R. sphaeroides*

on olive mill wastewater. Further, (Pintucci, 2014) has shown a COD decrease on olive mill wastewater using *R. palustris*.

(Wang *et al.,* 2007) showed successful textile effluent decolourization, up to 90 % with *R. palustris.* However, these conditions were optimised and therefore don't compare to the 52 % decolourization efficiency achieved in this study.

5.3.13. Industrial engineering aspect of this study

One of the major avenues for the destruction of toxic compounds contained in industrial wastewater is biodegradation. Biodegradation of a compound refers to the breakdown and elimination of a pollutant via the metabolic pathways of the living organism. This is generally achieved by bacteria that are naturally found in soil and water. Selection of the optimal treatment method for the purification of a specific industrial effluent is a highly complex objective. The suitability of biodegradation to a particular wastewater stream depends on the quality standards to be met and whether the most effective and efficient treatment is achieved with the lowest reasonable cost. It is important to avoid unnecessary expenditure of energy and chemicals, as this will lower the operating cost (Bandara *et al.,* 1997).

Consequently, the chief parameters which must be taken into consideration when deciding if a particular wastewater stream is suitable for biological wastewater treatment are the quality of the original wastewater, the removal of main contaminants, whether conventional treatment technologies are superior, the flexibility of the treatment if the original wastewater has a high degree of variation, the economics, the potential use of the decontaminated water, the time required for treatment and whether chemical pre-treatment is required (Radjenovic *et al.,* 2009).

Therefore, based on the above mentioned criteria, the wastewaters tested in this research would pose significant problems for industrial scale biological wastewater treatment. Many of them contain components in high concentrations which would inhibit the enzymatic and metabolic activity of *R. palustris* and possibly kill *R palustris*. To overcome this an acclimatization period would be required which would add additional time and costs to the overall process. *R. palustris* is also very sensitive to certain environmental conditions such as salinity, pH and oxygen concentration. Further, for many of the undiluted cases, low COD reductions were recorded which indicates that many of the main contaminants were not removed. The data also appeared to be inconsistent indicating that the obtained wastewater samples had high degrees of variation. It can be concluded that the results of this study would pose many obstacles for large scale wastewater treatment.

5.4. Wastewater treatment tests with immobilized cells

5.4.1. Batch immobilized winery wastewater treatment

The wastewater most suitable for *R. palustris* growth as well as wastewater treatment is winery wastewater as it doesn't need to be diluted. In addition, winery wastewater also performed well, strongly supporting bacterial growth and exhibiting large reductions to COD. Next, wastewater treatment tests were conducted again with winery wastewater, but this time with immobilized cells and not free cells. Cells were immobilized in PVA cryogels in order to prevent cell washout from the bio reactors which were operated continuously. The batch experiment was conducted to determine whether COD reduction was affected by the immobilization of cells. Figure 28 shows a comparison between the COD reduction achieved with immobilized cells for diluted and undiluted winery wastewater. Figure 29 shows a comparison between the COD achieved with free cells and immobilized cells for undiluted wastewater. Essentially, no difference was seen in the COD decrease for undiluted winery wastewater between free and immobilized cells with a 72 % and 70 % average decrease, respectively. This result is expected as the biomass concentration in the immobilized cell bioreactors is constant and the same as the final biomass concentration in the free cell bioreactors. Therefore, more biomass is in contact with the wastewater from the start of the experiment for the immobilized cells when compared to the free cells as there is no lag phase. For the free cell experiments, time is taken for the bacteria to grow and for the biomass to increase. However, for the immobilized cells, the COD reduction might be diffusion rate limited which will in turn result in a slower COD reduction when compared to the free cells.

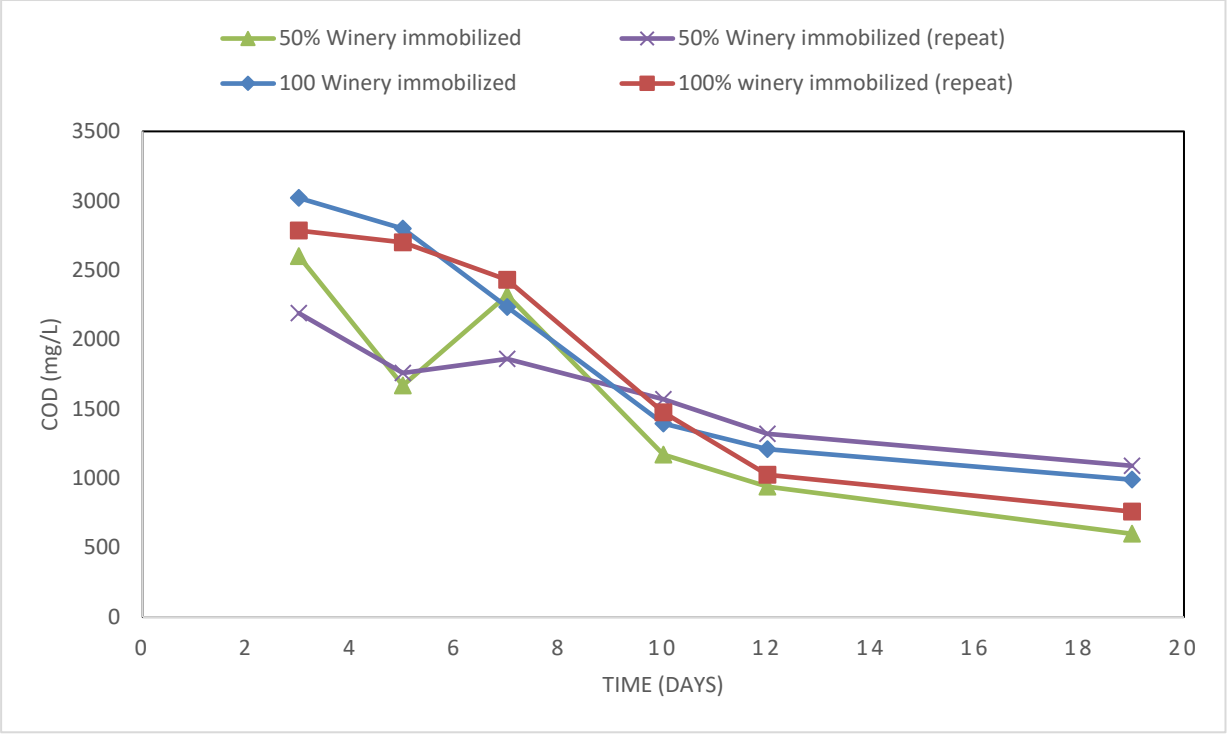

Figure *28: COD depletion of winery wastewater versus culture age – cells immobilized in PVA cryogel beads.*

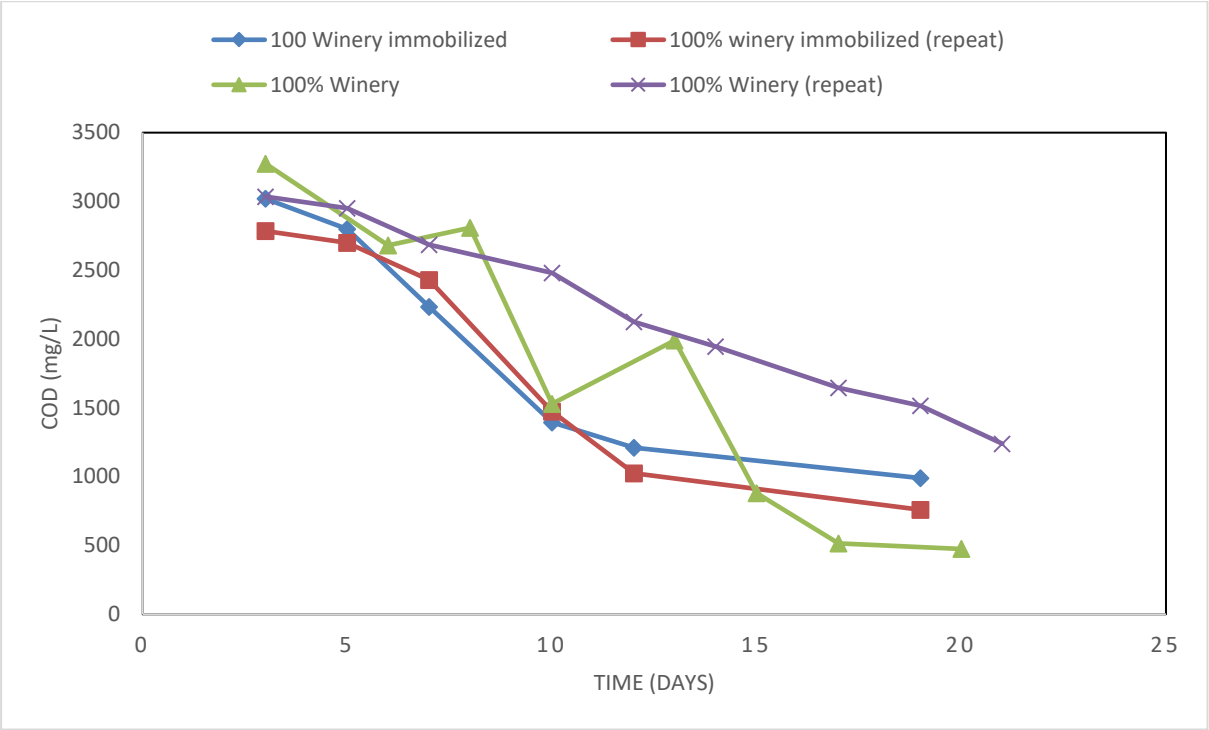

Figure *29: COD depletion of undiluted winery wastewater versus culture age – a comparison between immobilized and free cells.*

5.4.2. Continuous winery wastewater treatment results

Continuous wastewater treatment was carried out on undiluted winery wastewater.

The concentration of a liquid culture of *R. palustris* was determined to be 1.95 g cdw/L according to equation 10 in section 4.4.2.2. This liquid culture was centrifuged (20 minutes, 3000 RCF) in order to collect the biomass needed for immobilization. A total of 380 ml of liquid culture was centrifuged. The mass (cell dry weight) of biomass used for immobilization is calculated according to:

$$cell\ dry\ weight\ (g) = C\left(\frac{g\ cdw}{ml}\right) \times Volume\ (ml) \qquad \text{[Equation 13]}$$

$$cell\ dry\ weight\ (g) = 1.95 * 10^{-3}\left(\frac{g\ cdw}{ml}\right) \times 380\ (ml)$$

$$cell\ dry\ weight\ (g) = 0.741\ g\ cdw$$

This biomass was then immobilized in PVA cryogels according to the method described in section 4.5.

Figure 30 was constructed using data from a batch wastewater experiment on the winery wastewater. This experiment was conducted with free cells and shows the relationship between COD decrease and biomass increase over time. In other words, Figure 30 shows the COD depletion per biomass in the reactor over time. Figure 30 can be used to calculate the residence time and hence the flowrate required, under continuous operation, for a certain COD reduction when the concentration of biomass

in the reactor is known. A linear trendline is applicable to Figure 30 as the data very closely fits the regression line. Further, if the last two data points are ignored (when the biomass and COD concentration were essentially constant) the data will even more closely fit the regression line.

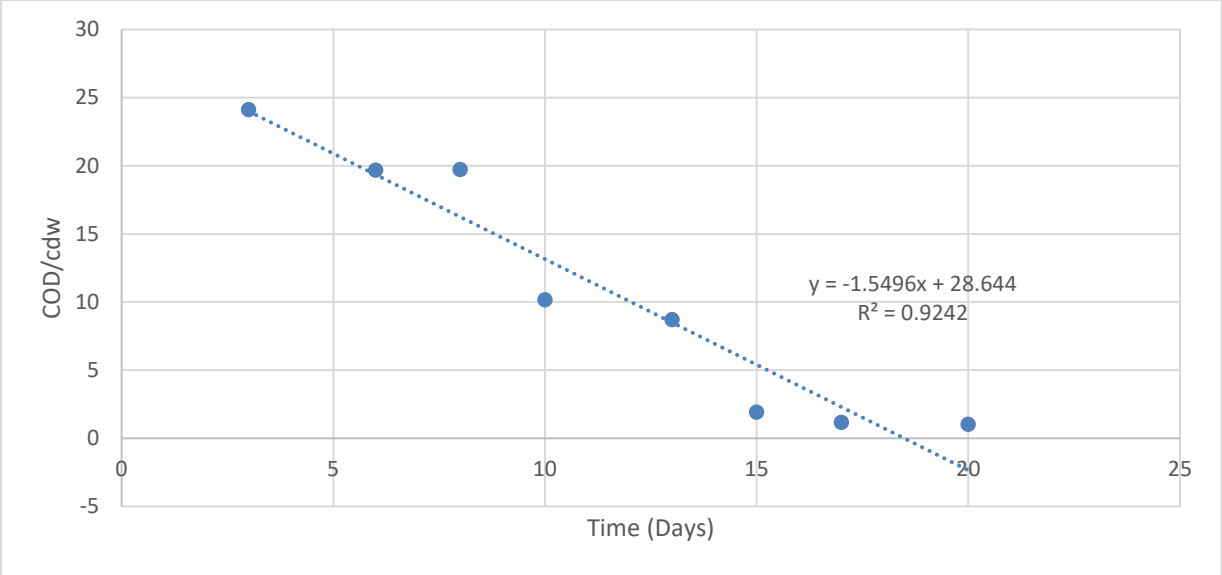

Figure *30: Specific COD consumption rate for free cells in a batch experiment on undiluted winery wastewater.*

With the winery wastewater having an initial COD of 3025 mg/L, and a target to decrease the COD by 5 % under continuous operation, that would mean a 151.25 mg/L (0.15125 g/L) reduction in COD. Further, half of the immobilized biomass (0.371 g cdw) was used for the continuous wastewater treatment experiment. This value (0.371 g cdw) compares well to the final biomass concentration which was present in the 100 % winery wastewater batch experiments (average is 0.417 g cdw). The equation of the trendline in Figure 30 is y = -1.5496x + 28.644. The gradient can be used to calculate the residence time and is expressed as follows:

$$-1.5496 = \frac{\Delta\frac{COD}{cdw}}{\Delta t} \qquad \text{[Equation 14]}$$

$$-1.5496 = \frac{\frac{-0.15125\ g/L}{0.371\ g/L}}{\Delta t}$$

$$\Delta t = 0.263\ days$$

$$\Delta t = 378.72\ minutes$$

The volume of the continuously operated photo-bioreactor was 500 ml. The cryogel beads occupy approximately 100 ml of the reactor volume leaving an effective operating volume of 400 ml. The flowrate required for a residence time of 378.72 minutes was calculated as follows:

$$\tau\ (minutes) = \frac{Volume\ (ml)}{Flowrate\left(\frac{ml}{\min}\right)}$$ [Equation 15]

$$378.72\ minutes = \frac{400\ ml}{Flowrate\left(\frac{ml}{\min}\right)}$$

$$Flowrate\ \left(\frac{ml}{min}\right) = 1.06\frac{ml}{minute} \approx 1\frac{ml}{minute}$$

Figure 31 was generated when winery wastewater was treated under continuous operation for 28 days at a flowrate of 1 ml/minute. Figure 31 shows the COD removal achieved compared to the desired COD removal of 5 %. 5 % was selected based off the data from batch experiments with winery wastewater. Taking into account the extremely long residence times required to achieve COD decreases greater than 5 %, and the desire to operate the bioreactor continuously, a conservative COD reduction was chosen.

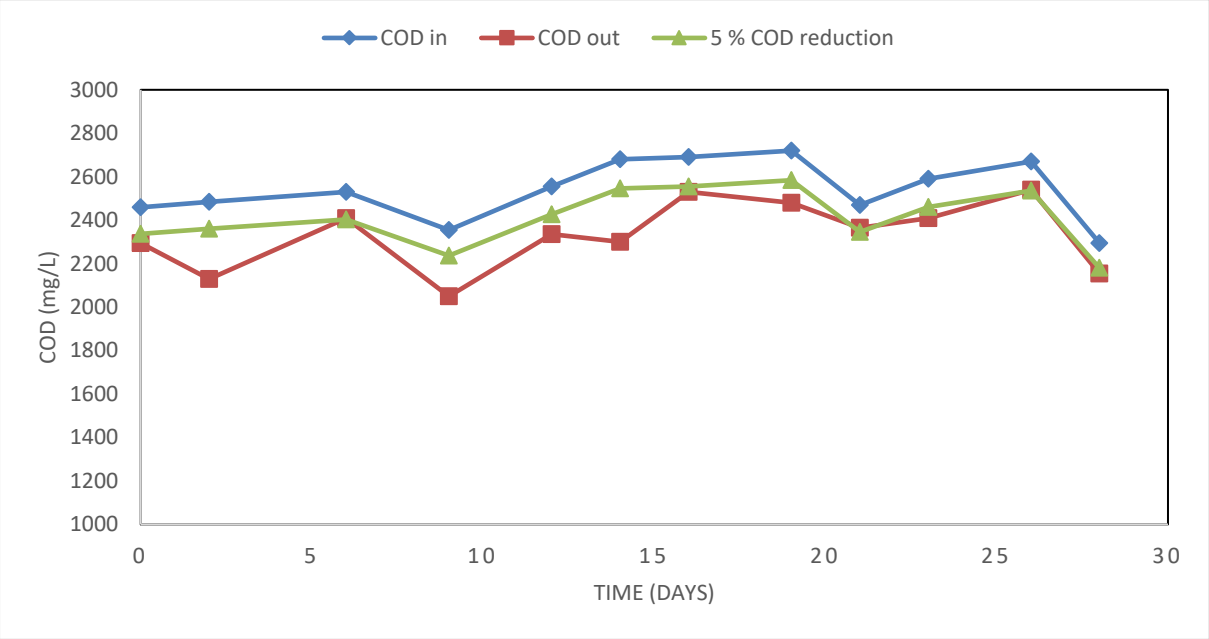

Figure *31: Performance of the bioreactor when operated continuously with undiluted winery wastewater.*

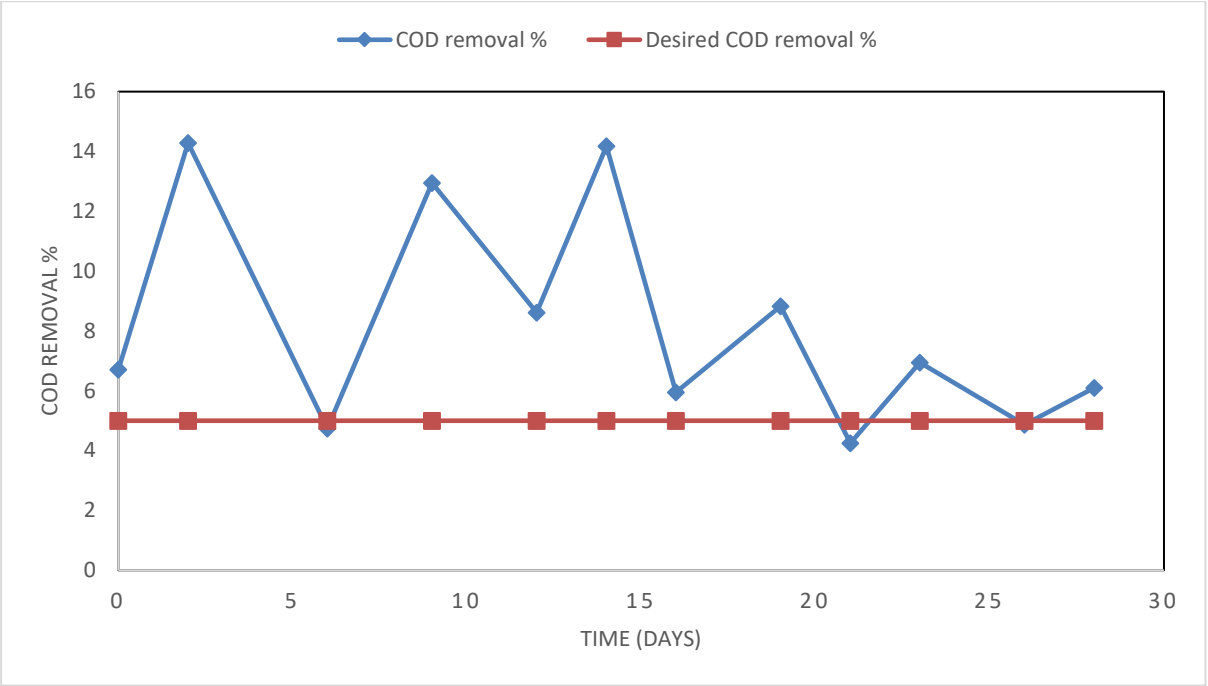

Figure *32: COD removal % of undiluted winery wastewater under continuous wastewater treatment.*

Analysis of Figure 32 shows that initially the COD removal % was substantially greater than the target of 5 %. This was due to a vacuum which formed in the continuous system resulting in decreased flowrates and increased residence times. This is reflected numerically where the average COD decrease is 10.25 % over the first 14 days and 6.16 % over the last 14 days. Overall, 29 L of winery wastewater was treated over 28 days. This results in an overall flowrate calculated as follows:

$$Flowrate\ \left(\frac{ml}{minute}\right) = \frac{Volume\ (ml)}{Time\ (minutes)} \qquad \text{[Equation 16]}$$

$$Flowrate\ \left(\frac{ml}{minute}\right) = \frac{29000\ ml}{40320\ minutes}$$

$$Flowrate\ \left(\frac{ml}{minute}\right) = 0.72\ ml/minute$$

5.5. Hydrogen production

Attempts to produce hydrogen through photo-fermentation of the various industrial wastewaters proved to be unsuccessful. Therefore, an experiment was conducted using glycerol as the carbon source and glutamate as the nitrogen source in order to demonstrate photo-fermentative hydrogen production under ideal conditions and with a synthetic waste stream. Figure 33 shows the cumulative biogas production over time. The procedure for this experiment was the same as those conducted with wastewater. The procedure is given in section 4.4.4.

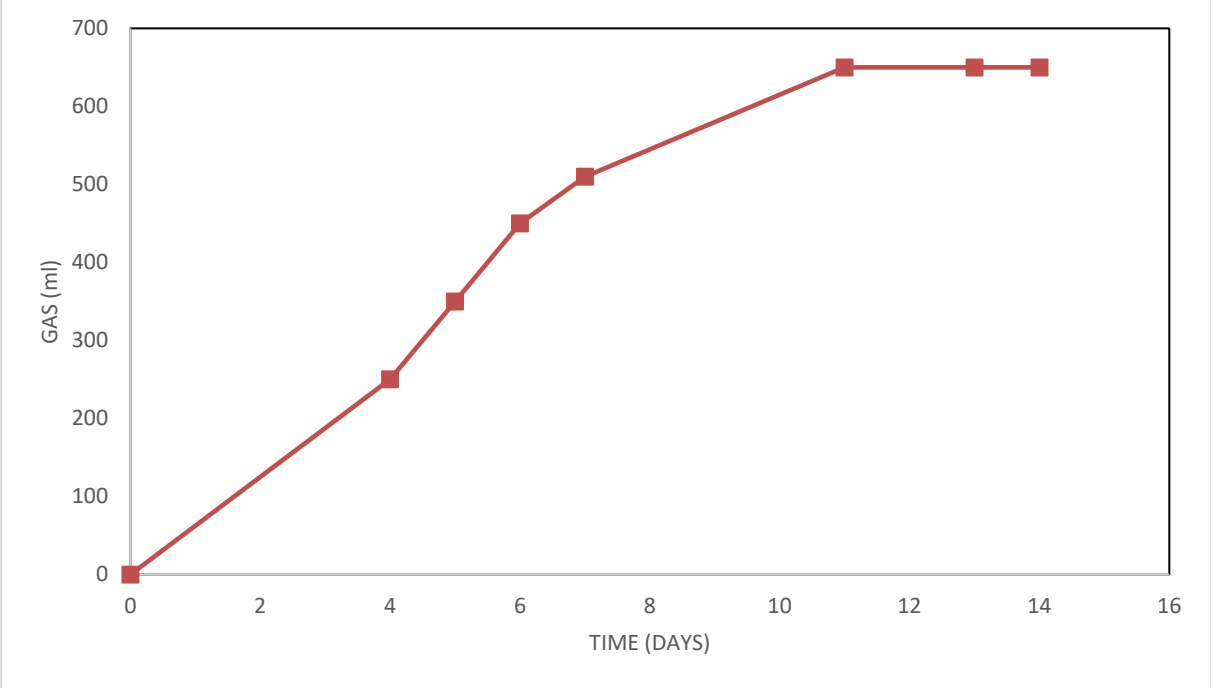

Figure *33: Cumulative gas production of R. palustris grown on glycerol (5M) and glutamate (2M).*

It is assumed that the composition of produced gas does not change over the 14 day period and that only hydrogen and carbon dioxide are produced. Therefore, the volumes of produced gas were recorded over the duration of the experiment and an analyses was conducted on the total gas produced. The gas chromatography (GC) results of the analysed biogas can be seen in Appendix D. For the experimental run shown in Figure 33, the gas consisted of 94.1 % hydrogen and 5.9 % carbon dioxide. Sample calculations for these procedures can be seen in Appendix G. For a repeat experimental run (graph not shown), the gas had the same hydrogen and carbon dioxide composition. This section shows that hydrogen can be produced under the correct experimental conditions.

6. CONCLUSIONS

The experimental work completed throughout this research arose due to the need for high organic content industrial wastewater treatment. A survey of local literature was conducted which brought to light the South African industries producing waste streams with a high organic content. This was followed by an assessment of these waste streams which determined which would be suitable for photo-fermentative treatment by *R. palustris*. The ability of *R. palustris* to metabolize a variety of waste organic components was evaluated.

The survey included the malt brewing, metal finishing, soft drink, sorghum beer, edible oil, red meat abattoir, laundry, tanning and leather finishing, paper and pulp, iron and steel, cane sugar processing, textile and poultry industries. Based on the key wastewater components contained within each industry's wastewater, an evaluation was made as to the photo-fermentative treatment potential. Effluents considered to be treatable through photo-fermentation were selected for experimental runs. These included winery, AD effluent, brewery, vinasse waste, olive processing, tannery, fish processing, paper and pulp and dye wastewater.

For each wastewater, a run was conducted with 100% wastewater as well as at increasing dilution rates with media. The media contained no carbon and no nitrogen source. For cases where no growth was observed, higher dilution rates were used (10 % wastewater in media). Table 8 shows the recommended dilution for each wastewater sample and provides a summary of *R. palustris* growth and COD removal at the recommended dilution. For the majority of the wastewaters, the growth appeared to be limited by the depletion of essential nutrients required for growth and not due to the wastewater containing toxic and harmful components. For cases when growth was not observed, the bacterial population was still able to metabolize the wastewater components as seen by a COD decrease.

Table 8: Summary of R. palustris growth on wastewater and corresponding COD decrease of the wastewater at the recommended dilution rate.

Wastewater	Recommended dilution	Growth	COD decrease
Winery	0 %	Yes	Yes
Ad effluent	90 %	Yes	Yes
Brewery	90 %	Yes	Yes
Vinasse	99 %	Poor	Yes
Olive processing	50 %	Yes	Yes
Tannery	50 %	Yes	Yes
Fish processing	50 %	Poor	Yes
Paper and pulp	50 %	Poor	Poor

For dye wastewater, an alternative approach was taken to monitor pollutant levels. The UV-VIS spectra of the dye wastewater was analysed. This was done in order to determine absorbance peaks which are caused by the presence of dye chemicals. The absorbance at the wavelengths at which the peaks occur was then tracked over a 23 day period. 50 % dye wastewater showed improved decolourization efficiencies when compared to 100 % dye wastewater. For 50 % dye wastewater at 288 nm (the wavelength at which aromatic amines absorb), a 52 % decolourization efficiency was achieved.

The wastewater most suitable for photo-fermentative wastewater treatment is winery wastewater as it doesn't need to be diluted yet exhibited good growth and a significant COD decrease. Next, wastewater treatment tests were conducted again with winery wastewater, but this time with immobilized cells and not free cells. Cells were immobilized in PVA cryogels so that continuous wastewater treatment could be evaluated, and would be comparable to conventional industrial wastewater treatment processes. It can be concluded that COD reduction of winery wastewater is not affected by the immobilization of the cells. Finally, a consistent COD removal was achieved over a 28 day period when the bioreactor was operated continuously.

The maximum specific COD consumption rate and the duration into the experiment at which they occur can be seen in Table 9. Despite observing growth and a COD reduction on the majority of the wastewaters, the time taken to reach the maximum specific COD consumption rate, and therefore the overall COD reduction was lengthy, and commonly longer than current alternative wastewater treatment technologies (Oller, Malato and Sánchez-Pérez, 2011). However, the COD reduction rate can be increased through various methods. These include but are not limited to increasing the biomass concentration as well as the illumination.

Furthermore, the metabolic advantages of *R. palustris* are twofold. Firstly, they can grow in wastewaters with high organic loading and they can reduce the COD content of the wastewater. Secondly, this process can also be modified to produce both hydrogen gas and bacterial biomass which can be used as an agricultural feedstock in the poultry and aquaculture industries (Getha, Vikineswary and Chong, 1998). Therefore, in spite of the lengthy COD reduction, *R. palustris* remains a candidate organism for wastewater treatment owing to the possible production of valuable commodity products.

Table 9: Maximum specific COD consumption rates and the duration into the experiment at which they occurred.

Wastewater	Maximum specific COD consumption rate (g COD/g biomass)	Time (Days)
100 % Winery	5.04	11
50 % Winery	5.18	9
10 % AD effluent	6.11	15
10 % Brewery	7.73	10
1 % Vinasse	224.85	12
100 % Olive processing	1.32	11.5
50 % Olive processing	1.70	13
100 % Tannery	0.76	7
50 % Tannery	1.25	6
100 % fish processing	0.96	13
50 % Fish processing	1.27	7
100 % Paper and pulp	3.35	7
50 % paper and pulp	4.50	5

In conclusion, this was a scoping study which focussed on the photo-fermentative treatment of organic containing industrial wastewaters without any pre-treatment steps. In other words, experiments were conducted on effluents in the state that they are discharged from the process plants from which they originate. Bacterial growth, COD removal and hydrogen production can be optimised for these effluents if the appropriate pre-treatment steps are employed.

With South Africa's abundance of wastewater streams and an ever increasing concern for water reuse and valorisation technologies, this bioprocess is considered a highly competitive water treatment technology for the removal of organic pollutants.

7. RECOMMENDATIONS

A number of recommendations can be made which will translate into improvements to the experimental procedures as well as the results generated within this research report. Firstly, *R. palustris* should be acclimatized to the various wastewaters before experiments are conducted. As far as is possible, experimental runs should be inoculated from bacteria grown in the same wastewater as the experimental run. This should accelerate microbial growth which will in turn result in a greater COD reduction.

Further wastewater producing industries should be investigated. These include but are not limited to the petroleum and petrochemical industries, food and beverage industries, landfill leachate, wastewater containing pesticides and herbicides and wastewater containing pharmaceuticals and emerging contaminants.

Additionally, experiments should be conducted where pH balancing of the wastewater is done prior to inoculation. For a number of the wastewaters, high dilutions rates had to be employed before growth was observed. This was not due to the wastewater containing components in concentrations which inhibit growth but due to low pH values of the wastewater. Therefore, if the pH of the wastewaters is increased to suit *R. palustris*, dilution with growth media might not be necessary. This may result in large savings in operating expenses if commercial wastewater treatment is considered.
For wastewaters with complex compositions and in which only a portion of the COD is reduced, a consortium of bacteria should be investigated in order to achieve a complete COD removal.

As it stands, no hydrogen has been produced from the various wastewaters and this is due to the presence of nitrogenous compounds in the wastewater. Hydrogen is concomitantly produced during nitrogenase activity, and nitrogenase activity is inhibited by the presence of nitrogenous compounds. A solution could be to use a nitrogen insensitive strain of *R. palustris* where the nitrogenase enzyme will still be active despite nitrogenous compounds. In addition, the experimental duration should be increased to the point of depletion of the nitrogenous compounds in order to determine if hydrogen production will start at this point.

Wastewater treatment tests should be conducted in a novel bioreactors in order to simulate industrial processes. If successful, this research will contribute towards wastewater treatment technologies which can impact both industrial and municipal levels through its application to recalcitrant organic wastes.

8. REFERENCES

Abo-Hashesh, M., Ghosh, D., Tourigny, A., Taous, A., & Hallenbeck, P. C. (2011). Single stage photofermentative hydrogen production from glucose: An attractive alternative to two stage photofermentation or co-culture approaches. *International Journal of Hydrogen Energy*, *36*(21), 13889–13895. https://doi.org/10.1016/j.ijhydene.2011.02.122

Ally, H., Kemish, W. and Van Der Spuy, T. (2016) *Water and Wastewater Management in the Metal Finishing Industry (Edition 2)*.

Boran, E., Özgür, E., Yücel, M., Gündüz, U., & Eroglu, I. (2012). Biohydrogen production by Rhodobacter capsulatus in solar tubular photobioreactor on thick juice dark fermenter effluent. *Journal of Cleaner Production*, *31*, 150–157. https://doi.org/10.1016/j.jclepro.2012.03.020

Chen, C. Y., Yang, M. H., Yeh, K. L., Liu, C. H., & Chang, J. S. (2008). Biohydrogen production using sequential two-stage dark and photo fermentation processes. *International Journal of Hydrogen Energy*, *33*(18), 4755–4762. https://doi.org/10.1016/j.ijhydene.2008.06.055

Cheng, J., Su, H., Zhou, J., Song, W., & Cen, K. (2011). Hydrogen production by mixed bacteria through dark and photo fermentation. *International Journal of Hydrogen Energy*, *36*(1), 450–457. https://doi.org/10.1016/j.ijhydene.2010.10.007

Crabtree, G. W., Dresselhaus, M. S. and Buchanan, M. V (2004) 'The Hydrogen Economy', *Physics Today*, (December), pp. 39–45. doi: 10.1063/1.1878333.

Das, D. (2001) 'Hydrogen production by biological processes: a survey of literature', *International Journal of Hydrogen Energy*, 26(1), pp. 13–28. doi: 10.1016/S0360-3199(00)00058-6.

DEA (2015) 'EIA Guideline for Renewable Energy Projects'.

Eroğlu, E., Eroğlu, I., Gündüz, U., Türker, L., & Yücel, M. (2006). Biological hydrogen production from olive mill wastewater with two-stage processes. *International Journal of Hydrogen Energy*, *31*(11), 1527–1535. https://doi.org/10.1016/j.ijhydene.2006.06.020

Eroğlu, E., Gündüz, U., Yücel, M., Türker, L., & Eroğlu, I. (2004). Photobiological hydrogen production by using olive mill wastewater as a sole substrate source. *International Journal of Hydrogen Energy*, *29*(2), 163–171. https://doi.org/10.1016/S0360-3199(03)00110-1

FASCETTI, E. (1998) 'Photosynthetic hydrogen evolution with volatile organic acids derived from the fermentation of source selected municipal solid wastes', *International Journal of Hydrogen Energy*, 23(9), pp. 753–760. doi: 10.1016/S0360-3199(97)00123-7.

Fu, Y. and Viraraghavan, T. (2001) 'Fungal decolorization of dye wastewaters: A review', *Bioresource Technology*, 79(3), pp. 251–262. doi: 10.1016/S0960-8524(01)00028-1.

Getha, K., Vikineswary, S. and Chong, V. C. (1998) 'Isolation and growth of the phototrophic bacterium *Rhodopseudomonas palustris* strain B1 in sago-starch-processing wastewater', *World Journal of Microbiology and Biotechnology*, 14(4), pp. 505–511. doi: 10.1023/A:1008855125634.

Gogate, P. R. and Pandit, A. B. (2004) 'A review of imperative technologies for wastewater treatment I: Oxidation technologies at ambient conditions', *Advances in Environmental Research*, 8(3-4), pp. 501–551. doi: 10.1016/S1093-0191(03)00032-7.

Hallenbeck, P. C. and Liu, Y. (2016) 'Recent advances in hydrogen production by photosynthetic bacteria', *International Journal of Hydrogen Energy*. Elsevier Ltd, 41(7), pp. 4446–4454. doi: 10.1016/j.ijhydene.2015.11.090.

Hassan, C. M. and Peppas, N. a (2000) 'Structure and Applications of Poly (vinyl alcohol) Hydrogels Produced by Conventional Crosslinking or by Freezing / Thawing Methods', *Adv. Polym. Sci.*, 153, pp. 37–65. doi: 10.1007/3-540-46414-X_2.

Ipcc (2007) *Climate Change 2007: impacts, adaptation and vulnerability: contribution of Working Group II to the fourth assessment report of the Intergovernmental Panel*, *Genebra, Suíça*. doi: 10.1256/004316502320517344.

Kapdan, I. K. and Kargi, F. (2006) 'Bio-hydrogen production from waste materials', *Enzyme and Microbial Technology*, 38(5), pp. 569–582. doi: 10.1016/j.enzmictec.2005.09.015.

Keskin, T., Abo-Hashesh, M. and Hallenbeck, P. C. (2011) 'Photofermentative hydrogen production from wastes', *Bioresource Technology*. Elsevier Ltd, 102(18), pp. 8557–8568. doi: 10.1016/j.biortech.2011.04.004.

Keskin, T. and Hallenbeck, P. C. (2012) 'Hydrogen production from sugar industry wastes using single-stage photofermentation', *Bioresource Technology*. Elsevier Ltd, 112, pp. 131–136. doi: 10.1016/j.biortech.2012.02.077.

Kim, M. S., Baek, J. S. and Lee, J. K. (2006) 'Comparison of H2 accumulation by Rhodobacter sphaeroides KD131 and its uptake hydrogenase and PHB synthase deficient mutant', *International Journal of Hydrogen Energy*, 31(1), pp. 121–127. doi: 10.1016/j.ijhydene.2004.10.023.

Kocyigit, H. and Ugurlu, A. (2015) 'Biological decolourization of reactive azo dye by anaerobic/aerobic sequencing batch reactor system', *Global NEST journal*, 17(X), pp. 1–10.

Lee, C. M., Chen, P. C., Wang, C. C., & Tung, Y. C. (2002). Photohydrogen production using purple nonsulfur bacteria with hydrogen fermentation reactor effluent. *International Journal of Hydrogen Energy*, *27*(11-12), 1309–1313. https://doi.org/10.1016/S0360-3199(02)00102-7

Lucas, M. S., Peres, J. A. and Li Puma, G. (2010) 'Treatment of winery wastewater by ozone-based advanced oxidation processes (O3, O3/UV and O3/UV/H2O2) in a pilot-scale bubble column reactor and process economics', *Separation and Purification Technology*, 72(3), pp. 235–241. doi: 10.1016/j.seppur.2010.01.016.

Maeda, I., Chowdhury, W. Q., Idehara, K., Yagi, K., Mizoguchi, T., Akano, T., … Miura, Y. (1998). Improvement of substrate conversion to molecular hydrogen by three-stage cultivation of a photosynthetic bacterium, Rhodovulum sulfidophilum. *Applied Biochemistry and Biotechnology*, *70-72*, 301–310. https://doi.org/10.1007/BF02920146

Van Der Merwe-Botha, M., Steytler, B. and Wille, P. (2017a) *Water and Wastewater Management in the Iron and Steel Industry*.

Van Der Merwe-Botha, M., Steytler, B. and Wille, P. (2017b) *Water and Wastewater Management in the Paper and Pulp Industry*.

Müller, J. (2017) *Water and Wastewater Management in the Red Meat Abattoir Industry (Edition 2)*.

Musee, N. (2016) *Water and Waste-Water Management in the Sorghum Beer Industry*.

Mutharasaiah, K., Govindareddy, V. and Chandrakant, K. (2012) 'Biodegradation of 2-Chlorophenol by *Rhodopseudomonas palustris*', *Bioremediation Journal*, 16(1), pp. 1–8. doi: 10.1080/10889868.2011.628348.

Nagadomi, H., Kitamura, T., Watanabe, M., & Sasaki, K. (2000). Simultaneous removal of chemical oxygen demand (COD), phosphate, nitrate and H2S in the synthetic sewage wastewater using porous ceramic immobilized photosynthetic bacteria. *Biotechnology Letters*, *22*(17), 1369–1374. https://doi.org/10.1023/A:1005688229783

Nath, K., Muthukumar, M., Kumar, A., & Das, D. (2008). Kinetics of two-stage fermentation process for the production of hydrogen. *International Journal of Hydrogen Energy*, *33*(4), 1195–1203. https://doi.org/10.1016/j.ijhydene.2007.12.011

'NATIONAL WASTE INFORMATION BASELINE REPORT' (2012).

Oda, Y., De Vries, Y. P., Forney, L. J., & Gottschal, J. C. (2001). Acquisition of the ability for Rhodopseudomonas palustris to degrade chlorinated benzoic acids as the sole carbon source. *FEMS Microbiology Ecology*, *38*(2-3), 133–139. https://doi.org/10.1016/S0168-6496(01)00180-5

Oh, Y. K., Seol, E. H., Lee, E. Y., & Park, S. (2002). Fermentative hydrogen production by a new chemoheterotrophic bacterium Rhodopseudomonas palustris P4. *International Journal of Hydrogen Energy*, *27*(11-12), 1373–1379. https://doi.org/10.1016/S0360-3199(02)00100-3

Oller, I., Malato, S. and Sánchez-Pérez, J. A. (2011) 'Combination of Advanced Oxidation Processes and biological treatments for wastewater decontamination-A review', *Science of the Total Environment*. Elsevier B.V., 409(20), pp. 4141–4166. doi: 10.1016/j.scitotenv.2010.08.061.

Pintucci, C., Padovani, G., Giovannelli, A., Traversi, M. L., Ena, A., Pushparaj, B., & Carlozzi, P. (2015). Hydrogen photo-evolution by Rhodopseudomonas palustris 6A using pre-treated olive mill wastewater and a synthetic medium containing sugars. *Energy Conversion and Management*, *90*, 499–505. https://doi.org/10.1016/j.enconman.2014.11.045

Pocock, G. and Joubert, H. (2017) 'WATER AND WASTE WATER MANAGEMENT IN THE POULTRY (Edition 2) Deliverable 6 : Final Report Report to the Water Research Commission by', 8(January).

Pollution Research Group UKZN (2015) 'Water and Wastewater Management in the Soft Drink Industry'.

Pott, R. W. M. (2013) 'The Bioconversion of Waste Glycerol into Hydrogen by Rhodopseudomonas palustris', (September).

Ramukhwatho, F., Seetal, A. and Pienaar, H. (2016) *Water and Wastewater Management in the Malt Brewing Industry*.

Roes-hill, L. (2017) 'WATER RESEARCH COMMISSION PROJECT K5 / 2489 / 3 REVISION OF NATSURV 13 : WATER AND WASTEWATER MANAGEMENT IN THE TEXTILE INDUSTRY (EDITION 2) Cape Peninsula University of Technology', (February).

Singh, S. P., Srivastava, S. C. and Pandey, K. D. (1994) 'Hydrogen production by Rhodopseudomonas at the expense of vegetable starch, sugarcane juice and whey', *International Journal of Hydrogen Energy*, 19(5), pp. 437–440. doi: 10.1016/0360-3199(94)90020-5.

Swartz, C., Jackson-Moss, C., Rowswell, R., Mpofu, A., & Welz, P. (2017). *Water and Waste-Water Management in the Tanning and Leather Industry* (Vol. 2).

Swartz, C., Swanepoel, G., Welz, P., Muanda, C., & Bonga, A. (2017). *Water and Wastewater Management in the Laundry Industry*.

Tao, Y., He, Y., Wu, Y., Liu, F., Li, X., Zong, W., & Zhou, Z. (2008). Characteristics of a new photosynthetic bacterial strain for hydrogen production and its application in wastewater treatment. *International Journal of Hydrogen Energy*, *33*(3), 963–973. https://doi.org/10.1016/j.ijhydene.2007.11.021

Wang Xingzu, Cheng Xiang, Sun Dezhi, Q. H. (2007) 'Biodecolorization and partial mineralization of Reactive Black 5 by a strain of Rhodopseudomonas palustris', 19(4), pp. 1014–1019. doi: 10.1016/S1001-0742(08)62363-3.

Welz, P. and Ndobeni, A. (2017) *Water, Wastewater and Energy Management and Recommendations for Best Practice in the Cane Sugar Processing Industry*.

Welz, P., Le Roes-Hill, M. and Swartz, C. (2017) *NATSURV 6 Water and Wastewater Management in the Edible Oil Industry (Edition 2)*.

Xu, J., Feng, Y., Wang, Y., & Lin, X. (2013). Characteristics of purple nonsulfur bacteria grown under Stevia residue extractions. *Letters in Applied Microbiology*, *57*(5), 420–426. https://doi.org/10.1111/lam.12129

Yetis, M., Gündüz, U., Eroglu, I., Yücel, M., & Türker, L. (2000). Photoproduction of hydrogen from sugar refinery wastewater by Rhodobacter sphaeroides O.U. 001. *International Journal of Hydrogen Energy*, *25*(11), 1035–1041. https://doi.org/10.1016/S0360-3199(00)00027-6

Zhu, H., Suzuki, T., Tsygankov, A. A., Asada, Y., & Miyake, J. (1999). Hydrogen production from tofu wastewater by Rhodobacter sphaeroides immobilized in agar gels. *International Journal of Hydrogen Energy*, *24*(4), 305–310. https://doi.org/10.1016/S0360-3199(98)00081-0

9. APPENDIX A – LITERATURE TABLE

Table 10: A review of literature – rates of hydrogen gas production from various carbon substrates as well as by various photo-fermentative bacteria.

Carbon substrate used	**Wastewater medium (synthetic or real wastewater)**	**Was *R. palustris* the selected bacteria?**	**H_2 production**	**Water treatment potential**	**Reference**
Glucose	Synthetic medium	No (R. capsulatus JY91)	3 mol H2 per mol glucose	Not evaluated	Abo-Hashesh 2011
Glucose	Synthetic medium	Yes (P4)	29.9 mmol/g cell h	Not evaluated	Oh 2002
Glucose	Synthetic medium	No (R. sphaeroides)	75 mL/L	Yes (COD decrease)	Tao 2008
Glucose	Synthetic medium	No (R. capsulatus B100)	NA	Yes (COD decrease)	Tao 2008
Glucose	Synthetic medium	No (R. capsulatus ST410)	NA	Yes (COD decrease)	Tao 2008
Glucose	Synthetic medium	No (R. sphaeroides mutant)	12.6 mL H2/L h	Yes (COD decrease)	Tao 2008
Glucose	Synthetic medium	No (R. sphaeroides ZX5)	75 mL H2/L h	Yes (COD decrease)	Tao 2008
Glucose	Synthetic medium	No (R. sphaeroides KD131)	0.53 ml H2/mg-cdw	Not evaluated	Kim 2004

Glucose	Synthetic medium	No (R. sphaeroides KD131)	0.24 ml H2/mg-cdw	Not evaluated	Kim 2004
Glucose medium (control)	Synthetic medium	No (R. sphaeroides)	1.9 L H2/h m2 gel	Yes (not explicitly stated)	Zhu 1999
Glucose and fructose	Synthetic medium	Yes (6A)	11.8 mL/g(dw)/h	Yes (COD decrease)	Pintucci 2014
Sucrose	Synthetic medium	No (R. capsulatus B100)	NA	Yes (COD decrease)	Tao 2008
Sucrose	Synthetic medium	No (R. capsulatus ST410)	NA	Yes (COD decrease)	Tao 2008
Sucrose	Synthetic medium	No (R. capsulatus Z1)	NA	Yes (COD decrease)	Tao 2008
Sucrose	Synthetic medium	No (R. sphaeroides ZX5)	40 mL H2/L h	Yes (COD decrease)	Tao 2008
Sucrose	Synthetic medium	No (R. capsulatus JP91)	14 mol H2/mol sucrose	Not evaluated	Keskin 2012
Sucrose	Synthetic medium	No (R. sphaeroides KD131)	0.24 ml H2/mg-cdw	Not evaluated	Kim 2004
Sucrose	Synthetic medium	No (R. sphaeroides KD131)	0.49 ml H2/mg-cdw	Not evaluated	Kim 2004
Sucrose	Synthetic medium	No (R. sphaeroides)	40 mL/L	Yes (COD decrease)	Tao 2008

Glycerol	Synthetic medium	No (R. sphaeroides KD131)	0 ml H2/mg-cdw	Not evaluated	Kim 2004
Glycerol	Synthetic medium	No (R. sphaeroides KD131)	0.13 ml H2/mg-cdw	Not evaluated	Kim 2004
Xylose	Synthetic medium	No (R. sphaeroides)	70 mL/L	Yes (COD decrease)	Tao 2008
Fructose	Synthetic medium	No (R. sphaeroides)	61 mL/L	Yes (COD decrease)	Tao 2008
Arabinose	Synthetic medium	No (R. sphaeroides)	0 mL/L	Yes (COD decrease)	Tao 2008
Cellobiose	Synthetic medium	No (R. sphaeroides)	0 mL/L	Yes (COD decrease)	Tao 2008
Maltose	Synthetic medium	No (R. sphaeroides)	5 mL/L	Yes (COD decrease)	Tao 2008

Acetate	Synthetic medium	No (R. sphaeroides KD131)	0.13 ml H2/mg-cdw	Not evaluated	Kim 2004
Acetate	Synthetic medium	No (R. sphaeroides KD131)	0.46 ml H2/mg-cdw	Not evaluated	Kim 2004
Acetate	Synthetic medium	No (R. sphaeroides)	90 mL/L	Yes (COD decrease)	Tao 2008
Acetate	Synthetic medium	Rhodopseudomonas sp.	25.2 mL H2/L h	Yes (COD decrease)	Tao 2008
Acetate	Synthetic medium	Yes (P4)	NA	Yes (COD decrease)	Tao 2008
Acetate	Synthetic medium	No (R. capsulata)	45 mL H2/L h	Yes (COD decrease)	Tao 2008
Acetate	Synthetic medium	No (R. capsulatus B100)	NA	Yes (COD decrease)	Tao 2008
Acetate	Synthetic medium	No (R. capsulatus ST410)	NA	Yes (COD decrease)	Tao 2008
Acetate	Synthetic medium	No (R. sphaeroides ZX5)	90 mL H2/L h	Yes (COD decrease)	Tao 2008
Acetate	Synthetic medium	Rhodopseudomonas sp.	25.2 mL H2/L h	Not evaluated	Karapinar 2006
Acetate	Synthetic medium	Yes	2.2 mL H2/L h	Not evaluated	Karapinar 2006
Acetate	Synthetic medium	No (R. capsulata)	0.88 mL/h	Not evaluated	Karapinar 2006

Acetate	Fermentation products of glucose	No (R. sphaeroides O.U. 001)	1.5 - 1.72 mol H2/mol acetic acid	Not evaluated	Nath 2007
Malate	Synthetic medium	No (R. sphaeroides KD131)	0.80 ml H2/mg-cdw	Not evaluated	Kim 2004
Malate	Synthetic medium	No (R. sphaeroides KD131)	1.16 ml H2/mg-cdw	Not evaluated	Kim 2004
Malate	Synthetic medium	Rhodopseudomonas sp.	1.1 mL H2/L h	Yes (COD decrease)	Tao 2008
Malate	Synthetic medium	Yes	5.8 mL H2/L h	Yes (COD decrease)	Tao 2008
Malate	Synthetic medium	No (R. sphaeroides OU001)	5 mL H2/L h	Yes (COD decrease)	Tao 2008
Malate	Synthetic medium	No (R. capsulatus B100)	90 mL H2/L h	Yes (COD decrease)	Tao 2008
Malate	Synthetic medium	No (R. capsulatus ST410)	130 mL H2/L h	Yes (COD decrease)	Tao 2008
Malate	Synthetic medium	No (R. sphaeroides ZX5)	92 mL H2/L h	Yes (COD decrease)	Tao 2008
Malate	Synthetic medium	No (R. sphaeroides)	92 mL/L	Yes (COD decrease)	Tao 2008
Malate	Synthetic medium	Rhodopseudomonas sp.	1.1 mL H2/L h	Not evaluated	Karapinar 2006

Malate	Synthetic medium	Yes	5.8 mL H2/L h	Not evaluated	Karapinar 2006
Malate	Synthetic medium	No (R. sphaeroides)	12 mL/L h	Not evaluated	Karapinar 2006
L-malic acid	Synthetic medium	Yes	Not evaluated (growth evaluated)	Not evaluated	Xu 2013
Lactate	Synthetic medium	Rhodopseudomonas sp.	10.7 mL H2/L h	Yes (COD decrease)	Tao 2008
Lactate	Synthetic medium	Yes	9.1 mL H2/L h	Yes (COD decrease)	Tao 2008
Lactate	Synthetic medium	No (R. sphaeroides RV)	62.5 mL H2/L h	Yes (COD decrease)	Tao 2008
Lactate	Synthetic medium	No (R. sphaeroides ZX5)	103 mL H2/L h	Yes (COD decrease)	Tao 2008
Lactate	Synthetic medium	Rhodopseudomonas sp.	10.7 mL H2/L h	Not evaluated	Karapinar 2006
Lactate	Synthetic medium	Yes	9.1 mL H2/L h	Not evaluated	Karapinar 2006
Lactate	Synthetic medium	No (R. sphaeroides RV)	1.5 L/L d	Not evaluated	Karapinar 2006
Lactate	Synthetic medium	No (R. capsulatus IR3)	NA	Not evaluated	Karapinar 2006
Lactate	Synthetic medium	No (R. sphaeroides GL-1)	0.2 mL/mL PU matrix h	Not evaluated	Karapinar 2006
Lactate	Synthetic medium	No (R. sphaeroides)	103 mL/L	Yes (COD decrease)	Tao 2008

Lactate	Synthetic medium	No (R. sphaeroides KD131)	0.47 ml H2/mg-cdw	Not evaluated	Kim 2004
Lactate	Synthetic medium	No (R. sphaeroides KD131)	1.03 ml H2/mg-cdw	Not evaluated	Kim 2004
Butyrate	Synthetic medium	Rhodopseudomonas sp.	7.6 mL H2/L h	Not evaluated	Karapinar 2006
Butyrate	Synthetic medium	No (R. capsulata)	1.28 mL/h	Not evaluated	Karapinar 2006
Butyrate	Synthetic medium	No (R. sphaeroides)	118 mL/L	Yes (COD decrease)	Tao 2008
Butyrate	Synthetic medium	Rhodopseudomonas sp.	7.6 mL H2/L h	Yes (COD decrease)	Tao 2008
Butyrate	Synthetic medium	No (R. capsulata)	65 mL H2/L h	Yes (COD decrease)	Tao 2008
Butyrate	Synthetic medium	No (R. sphaeroides ZX5)	118 mL H2/L h	Yes (COD decrease)	Tao 2008
Butyric acid and acetic acid	Synthetic medium	Yes (WP3-5)	7.2 mol H2/mol sucrose	Yes (72 % COD reduction)	Chen 2008
Succinate	Synthetic medium	No (R. sphaeroides)	108 mL/L	Yes (COD decrease)	Tao 2008

Succinate	Synthetic medium	No (R. sulfidophilum)	26.6 mL H2/L h	Yes (COD decrease)	Tao 2008
Succinate	Synthetic medium	No (R. sphaeroides ZX5)	108 mL H2/L h	Yes (COD decrease)	Tao 2008
Succinate	Succinate wastewater	No (R. sphaeroides ZX5)	55 mL/L h	Yes (87.55 % COD removed)	Tao 2008
Succinate	Synthetic medium	No (R. sulfidophilum)	26.6 mL/L h	Not evaluated	Karapinar 2006
Succinate	Algal fermentative products	No (R. sulfidophilum)	34.3 µmol/mg cell dry wt	Not evaluated	Maeda 1998
Pyruvic acid	Synthetic medium	No (R. sphaeroides)	110 mL/L	Yes (COD decrease)	Tao 2008
Propionate	Synthetic medium	No (R. sphaeroides)	112 mL/L	Yes (COD decrease)	Tao 2008
Valeric acid	Synthetic medium	No (R. sphaeroides)	25 mL/L	Yes (COD decrease)	Tao 2008
Caproate	Synthetic medium	No (R. sphaeroides)	0 mL/L	Yes (COD decrease)	Tao 2008

Isovaleric acid	Synthetic medium	No (R. sphaeroides)	0 mL/L	Yes (COD decrease)	Tao 2008
Isobutyric acid	Synthetic medium	No (R. sphaeroides)	20 mL/L	Yes (COD decrease)	Tao 2008
Ethanol	Algal fermentative products	No (R. sulfidophilum)	0.46 µmol/mg cell dry wt	Not evaluated	Maeda 1998
Ethanol	Synthetic medium	No (R. sphaeroides)	0 mL/L	Yes (COD decrease)	Tao 2008
D-mannitol	Synthetic medium	No (R. sphaeroides)	75 mL/L	Yes (COD decrease)	Tao 2008
Benzoic acid	Synthetic medium	No (R. sphaeroides)	0 mL/L	Yes (COD decrease)	Tao 2008
Vanillic acid	Synthetic medium	No (R. sphaeroides)	0 mL/L	Yes (COD decrease)	Tao 2008

Starch	Synthetic medium	No (R. sphaeroides KD131)	0 ml H2/mg-cdw	Not evaluated	Kim 2004
Starch	Synthetic medium	No (R. sphaeroides KD131)	0.03 ml H2/mg-cdw	Not evaluated	Kim 2004
Carbohydrates	Olive wastewater	No (R. sphaeroides OU001)	NA	Yes (35.00 % COD removed)	Tao 2008
Carbohydrates	Olive wastewater	No (R. sphaeroides OU001)	NA	Yes (52.00 % COD removed)	Tao 2008
Carbohydrates	Olive mill wastewater	No (R. sphaeroides OU001)	4 mL/L culture h	Yes (COD decrease)	Karapinar 2006
Polyphenols, glucose, fructose, mannitol, acetic acid and malic acid but poor in VFA's	Pre-treated fresh olive mill wastewater diluted with water (30 % v:v)	Yes (6A)	13.5 mL/g(dw)/h	Yes (COD decrease)	Pintucci 2014
Organic matter and phenols	Olive mill wastewater (OMW)	No (R. sphaeroides O.U. 001)	13.9 L H2/L OMW	Yes (COD decreased from 1100 to 720 mg/L)	Eroglu 2004

Organic matter and phenols	Olive mill wastewater (OMW)	No (R. sphaeroides O.U. 001)	13.9 L H2/L OMW	Yes (BOD decreased from 475 to 200 mg/L)	Eroglu 2004
Organic matter and phenols	Olive mill wastewater (OMW)	No (R. sphaeroides O.U. 001)	13.9 L H2/L OMW	Total recoverable phenol content decreased from 2.32 to 0.93 mg/L	Eroglu 2004
Sugar refinery effluent + malic acid	Sugar refinery effluent	No (R. sphaeroides OU001)	5 mL/L culture h	Yes (COD decrease)	Karapinar 2006
Sugar refinery effluent + malic acid	Sugar refinery effluent	No (R. sphaeroides OU001)	3 mL/L culture h	Yes (COD decrease)	Karapinar 2006
Not given	Sugarcane juice	Rhodopseudomonas sp.	45 ml H2/g d w/h	Not evaluated	Fascetti 1998
Not given	Sugarcane wastewater	Rhodopseudomonas sp.	15 ml H2/g Bchl-a/h	Not evaluated	Fascetti 1998
Not given	Sugar refinery waste	Yes	30 ml H2/g d w/h	Not evaluated	Fascetti 1998
Not specified	Sugarcane juice	Rhodopseudomonas sp. BHU 1-4	45 µl H2/h mg bacterial cell dry weight	Not evaluated	Singh 1993
Reduced sugar, formic acid, acetic acid, oxalic	Simulated effluent of stevia residue	Yes	Not evaluated (growth evaluated)	Not evaluated	Xu 2013

acid, tartaric acid, malic acid, ascorbic acid, citric acid					
Water 16 %, sucrose 51 %, amino acids 8 %, organic acids 11 %, minerals 11.5 %	Beet molasses (sugar industry)	No (R. capsulatus JP91)	10.5 mol H2/mol sucrose	Not evaluated	Keskin 2012
Water 20 %, sucrose 32 %, glucose 15 %, fructose 16 %, amino acids 3 %, organic acids 7 %, minerals 8 %	Black strap (sugar industry)	No (R. capsulatus JP91)	8 mol H2/mol sucrose	Not evaluated	Keskin 2012
Sucrose + sugar refinery wastewater and L-malic acid + sugar refinery waste water	Pre-treated sugar refinery wastewater	No (R. sphaeroides O.U. 001)	0.005 l hydrogen/h/l culture	Not evaluated	Yetis 2000
Carbohydrates	Tofu wastewater	No (R. sphaeroides RV)	NA	Yes (41.00 % COD removed)	Tao 2008

Carbohydrates and proteins	Tofu wastewater	No (R. sphaeroides)	2.1 L/h m2 gel	Yes (COD decrease)	Karapinar 2006
Carbohydrates and proteins	Tofu wastewater	No (R. sphaeroides)	15.9 mL/L h	Yes (COD decrease)	Karapinar 2006
Starch 6750 mg/L, sucrose 800 mg/L, protein 630 mg/L, sugar reductive 250 mg/L, volatile acids 200 mg/L	Tofu wastewater	No (R. sphaeroides)	2.1 L H2/h m2 gel	Yes (TOC removal ratio in 85 h reached 41 %)	Zhu 1999
Glutamate-malate cultivation medium and glucose and peptone treatment conditions	Synthetic sewage wastewater	No (R. sphaeroides S, NR-3)	Not evaluated	Yes (removal of COD 89 %, phosphate 77 %, nitrate 99 % and H2S 99.8 %)	Nagadomi 2000
Glutamate-malate cultivation medium and glucose and peptone treatment conditions	Synthetic sewage wastewater	Yes	Not evaluated	Yes (removal of COD 89 %, phosphate 77 %, nitrate 99 % and H2S 99.8 %)	Nagadomi 2000

Glutamine or lactate	Textile effluent	Yes (W1)	Not evaluated	Yes (Reactive black 5 (RB5) decolourization)	Wang 2008
Yeast extract and peptone	Effluents of paper and wood industry	Yes	Not evaluated	Yes (*R. palustris* degraded about 97 % of the supplemented 2-CP	Mutharasaiah 2012
Not given	Straw paper mill effluent	Yes	30 ml H2/g d w/h	Not evaluated	Fascetti 1998
Butyrate and acetate	Effluents from dark fermentation of WW from a fuel ethanol manufacturer	No (R. sphaeroides ZX5)	48 mL/L h	Yes (84.55 % COD removed)	Tao 2008
Butyrate and acetate	Fermentation effluent from dark fermentation of kitchen waste	No (R. sphaeroides ZX5)	45 mL/L h	Yes (80.00 % COD removed)	Tao 2008

Acetate and butyrate	Soluble metabolite products of dark fermentation of cassava starch	Yes	489 ml H2/g starch (3.54 mol H2/mol hexose)	Not evaluated	Cheng 2010
Organic acids (and ammonia)	Effluent from hydrogen fermentation reactors (CSTR, peptone fed)	Yes (WP2-5 and WP3-5)	No (high ammonia conc. but growth possible)	Not evaluated	Lee 2002
Organic acids mainly butyric acid	Effluent from hydrogen fermentation reactors (ASBR, sugar fed)	Yes (WP2-5 and WP3-5)	Insignificant H2 produced due to presence of ammonia and ethanol	Not evaluated	Lee 2002
Organic acids mainly butyric acid	Effluent from hydrogen fermentation reactors (UASB, sugar fed)	Yes (WP2-5 and WP3-5)	Very low H2 prod due to use of carbonate as medium buffer which produced CO2	Not evaluated	Lee 2002
Not specified (volatile organic acids)	Effluent from hydrogen fermentation reactors (CSTR, sugar fed)	Yes (WP2-5 and WP3-5)	Yes but due to high ammonia conc. large amounts of H2 gas not produced	Not evaluated	Lee 2002

Not specified	Effluent from hydrogen fermentation reactors (CSTR, glucose and beef extract)	Yes (WP2-5 and WP3-5)	Yes (low ammonia conc. and phosphate used as medium buffer)	Not evaluated	Lee 2002
Not given	Lactic acid fermentation waste	No (Rhodobacter sphaeroides)	1.8 ml H2/g d w/h	Not evaluated	Fascetti 1998
Phenols and organic acids	Dark fermentation effluent from 50 % (v/v) activated sludge and olive mill wastewater	No (R. sphaeroides O.U. 001)	0.008 L/(L h)	Yes	Eroglu 2006
Phenols and organic acids	Effluent of clay pre-treatment process	No (R. sphaeroides O.U. 001)	Yes (not explicitly given)	Yes (52 % COD reduction)	Eroglu 2006
Acetic acid, phenol and ethanol (not consumed)	Thick juice dark fermenter effluent (DFE)	No (R. capsulatus)	0.15 mol H2/(m3 h)	Yes (68 % COD reduction)	Boran 2012
Not specified	Sago-starch processing decanter	Yes (B1)	Not evaluated (growth evaluated)	Yes (77 % COD reduction)	Getha 1998
Not given	Vegetable starch	Rhodopseudomonas sp.	30 ml H2/g d w/h	Not evaluated	Fascetti 1998

Not specified	Potato starch	Rhodopseudomonas sp. BHU 1-4	30 µl H2/h mg bacterial cell dry weight	Not evaluated	Singh 1993
Not specified	Whey	Rhodopseudomonas sp. BHU 1-4	25 µl H2/h mg bacterial cell dry weight	Not evaluated	Singh 1993
Not given	Whey	Rhodopseudomonas sp.	25 ml H2/g d w/h	Not evaluated	Fascetti 1998
Not given	Dairy wastewater	Rhodopseudomonas sp.	16 ml H2/g Bchl-a/h	Not evaluated	Fascetti 1998
Not given	Distillery wastewater	No (Rhodobacter sphaeroides)	10.4 ml H2/g d w/h	Not evaluated	Fascetti 1998
Lactic acid (90 %) as well as acetic acid, propionic acid and butyric acid	Lactate from municipal solid waste	No (Rhodobacter sphaeroides)	1.2 ml H2/g d w/h	Not evaluated	Fascetti 1998
Lactic acid (90 %) as well as acetic acid, propionic acid and butyric acid	Enriched lactate liquor	No (Rhodobacter sphaeroides)	100 ml H2/g d w/h	Not evaluated	Fascetti 1998

PHB	Synthetic medium	No (R. sulfidophilum)	33 mL/L h	Not evaluated	Karapinar 2006
Not given	Fermented cow dung	No (R. capsulata)	6.35 ml H2/g d w/h	Not evaluated	Fascetti 1998
Not given	Orange processing effluent	No (Rhodopseudomonas sphaeroides)	133 ml H2/g Bchl-a/h	Not evaluated	Fascetti 1998
Chlorinated benzoic acids - 3-chlorobenzoate (3-CBA) and benzoate (BA). Also 2-CBA and 4-CBA.	Synthetic medium	Yes	Not evaluated	Yes (degradation of 3-CBA if growth in a substrate containing BA)	Oda 2001

10. APPENDIX B – LIGHT SOURCE SELECTION

Incandescent light bulbs were chosen as light sources for *R. palustris* above fluorescent and halogen light bulbs. Incandescent light bulbs produce a continuous spectrum of light as they emit light via black body radiation. On the other hand, fluorescent light bulbs do not emit wavelengths light near the infrared region which *R. palustris* requires.

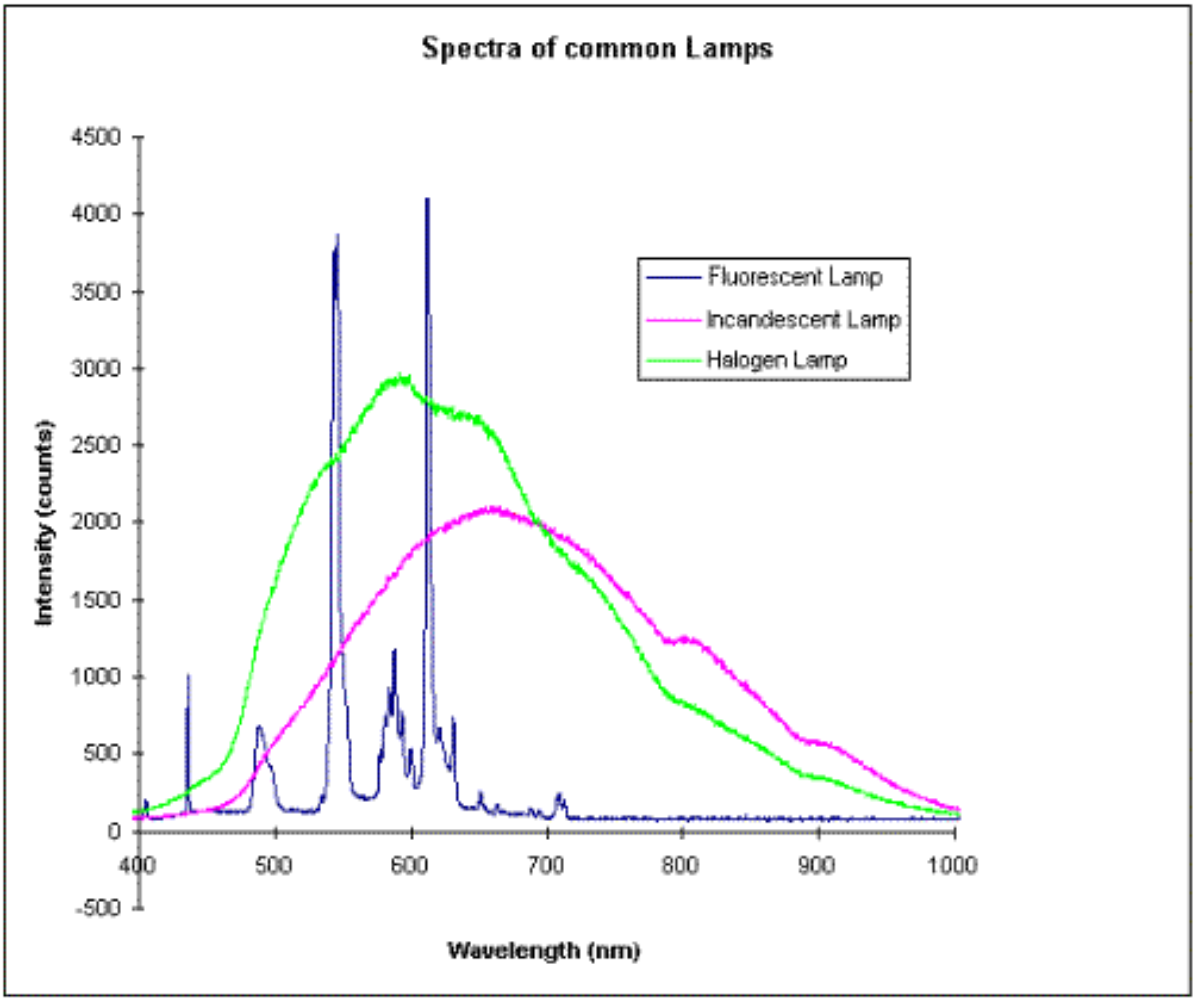

Figure *34: Spectra of common lamps.* Figure *reproduced from http://minerva.union.edu/newmanj/Physics100/Light%20Production/LampSpectra.gif.*

11. APPENDIX C – METABOLISM INVOLVED IN HYDROGEN PRODUCTION

Metabolism of *Rhodopseudomonas palustris*

- Preferred regime for growth and hydrogen production is **photoheterotrophism**.
- Illumination.
- Breakdown of organic molecules (e.g. organic acids) for use as both a source of carbon and of reducing potential.
- During respiration, the carbon compounds are often oxidized rather than assimilated, although in order to maintain redox poise carbon dioxide is fixed by the enzyme RuBisCo.
- However, when there is an inorganic electron donor present then the carbon source is primarily used for carbon assimilation rather than being oxidized to carbon dioxide.
- Under nitrogen limitation the nitrogen fixation pathway is expressed.
- The nitrogenases, which represent the major enzymatic constituent in this pathway, reduce atmospheric nitrogen to ammonia, with the concomitant obligate production of molecular hydrogen.
- Hydrogen production also serves as an electron sink, which the bacteria utilize to maintain redox poise by funneling excess energy and reducing potential into the production of molecular hydrogen.
- CBB pathway still in operation to maintain redox poise.
- Hydrogen production by *R. palustris* (and other PNS bacteria) occurs via two metabolic pathways.
- The first is through the biological water-gas shift reaction, whereby the bacterium utilizes the reducing power of carbon monoxide, under fermentative conditions, to produce hydrogen.
- The second pathway is photo-fermentation.
- This occurs under illumination in an anaerobic atmosphere and results in the breakdown of reduced substrates (organic acids).
- Culture should be under nitrogen limitation or the nitrogen source should be glutamic acid so as to stimulate the synthesis and activation of nitrogenase.

- Under these conditions the bacteria maintain redox poise by routing excess energy and reducing power to the nitrogenase-catalyzed production of hydrogen.
- Components of hydrogen production system: enzymes, photosystem and the Calvin-Benson-Bassham (CBB) cycle and storage compounds.

In Figure 35:

- Light absorbed by LH1 and LH2.
- RC used to drive a charge separation across cell membrane, where reducing equivalents are transported by ubiquinone (Q) and a proton gradient is created by cytochrome b/c_1 ($cytbc_1$).
- Proton gradient utilized by ATPase to produce ATP.
- Photosystem operates a 'cyclic' electron transport chain (purple arrows) as there is no terminal electron acceptor and so electrons are cycled back to the light harvesting complex.
- A flux of reducing equivalents leaves the ubiquinone pool (black straight arrows).
- Reducing equivalents leaving the cyclic transport chain, transported by ferredoxin, are used by nitrogenase to fix dinitrogen into ammonia, with the concurrent obligate production of hydrogen.
- Some of this hydrogen is hydrolyzed back to electrons and protons by an uptake hydrogenase (Hup).
- Reducing potential is also used by the cell to fix CO_2 in the CBB pathway (Calvin cycle) and store the carbon as poly(hydroxybutrate) (PHB) and glycogen.
- The TCA cycle (Krebs cycle) makes up the electrons lost to the photosystem by metabolizing organic substrates.

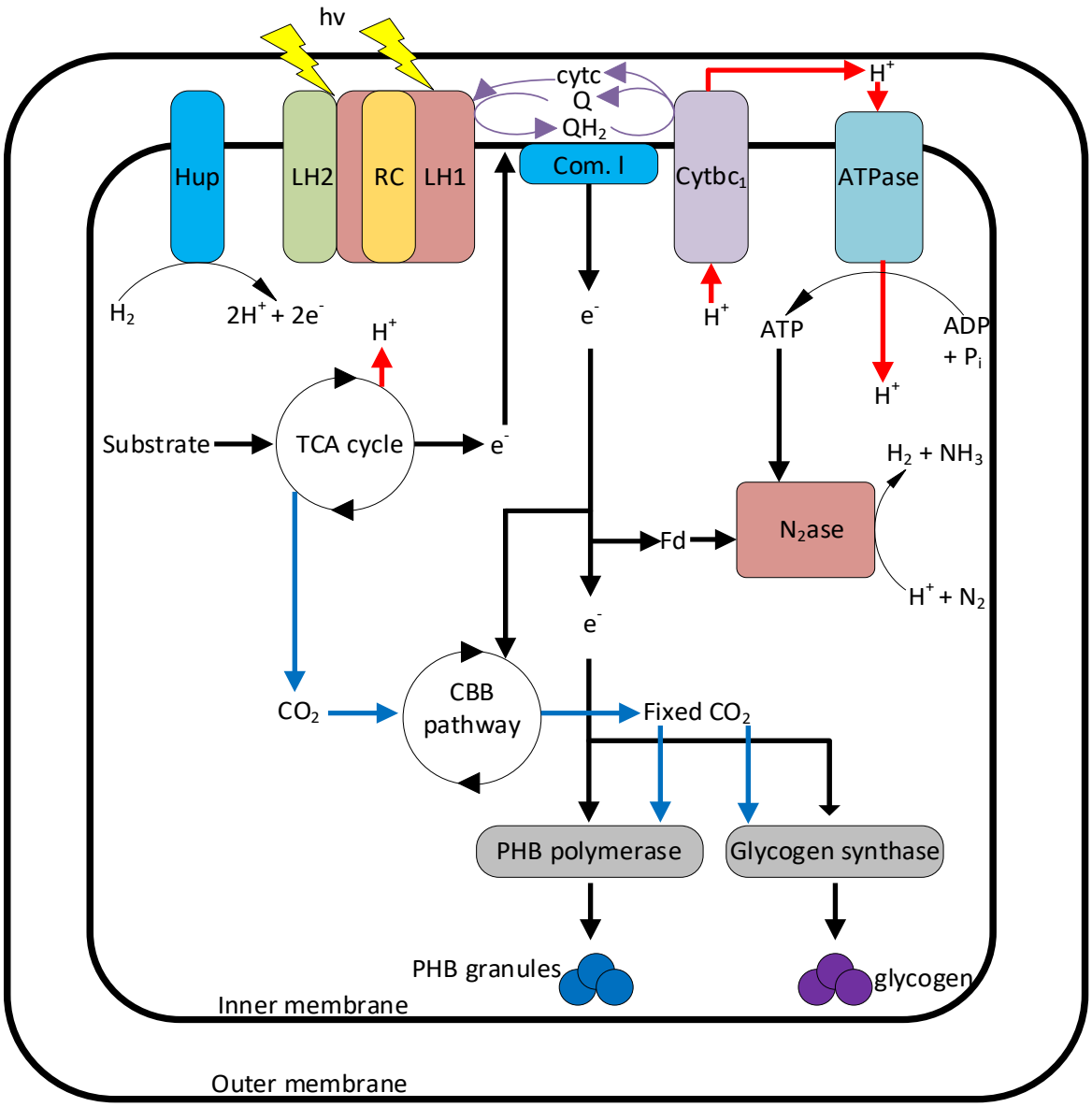

Figure *35: Main processes related to hydrogen metabolism in R. palustris under photoheterotrophic, anaerobic and nitrogen fixing conditions (Pott, 2013).*

- Straight black arrows: electron flow, through electron transporter flow.
- Straight blue arrows: CO_2 or fixed carbon flow.
- Straight red arrows: H^+ flow.
- Curved black arrows: reactions.
- Curved purple arrows: cyclic electron transport system, as part of the photosynthetic apparatus.
- Yellow lightning: light excitation.

12. APPENDIX D – GAS CHROMATOGRAPHY RESULTS

Table 11: Gas chromatography results of gas produced by R. palustris using glycerol as carbon source and glutamate as nitrogen source.

No.	Injection Name	Retention (DETECTED)	Amount	Retention (DETECTED)	Amount
		min	%	min	%
		TCD_Channel_2	TCD_Channel_2	TCD_Channel_3	TCD_Channel_3
		CO2	CO2	H2	H2
	PRS-R1-12102017	0.93	1.1413	0.79	18.176
	PRS-R2-12102017	0.93	0.7577	0.79	12.056

13. APPENDIX E – WASTEWATER ANALYSIS RESULTS

Table 12: Water analysis report from Bemlab for olive processing wastewater, brewery wastewater, winery wastewater and AD effluent.

Sampled by P. Uys

Water Analyses Report

Origin	Lab. Nr.	pH @ 25°C	EC @ 25°C mS/m	Na mg/l	K mg/l	Ca mg/l	Mg mg/l	B mg/l	P mg/l	NH_4-N mg/l	NO_3-N mg/l	*NO_2-N mg/l	*F mg/l	COD mg/l	*Hg µg/l	Date Sampled	*N mg/l	*Suspended solids (mg/l)
Olive WW	18129	5.8	59	66.9	31.7	28.0	11.7	<0.08	1.14	0.29	<0.36	0.65	0.0	2850.00	<3.1	Unknown	20.00	841
Brew WW	18130	4.2	240	43.7	110.7	327.5	182.8	<0.08	216.92	22.86	<0.36	1.65	0.0	20200.00	<3.1	Unknown	48.00	2491
Winery WW	18131	4.4	25	14.2	30.9	12.4	3.5	<0.08	2.01	0.66	<0.36	0.14	0.0	3045.00	<3.1	Unknown	8.00	223
AD WW	18132	5.7	1455	989.1	640.5	1383.6	120.7	1.70	71.78	355.47	<0.36	1.63	0.0	32950.00	<3.1	Unknown	50.00	1776

Origin	Lab. Nr.	Temperature at reception (°C)	*S A R	*PO_4 mg/l	*TOC mg/l	*Cl_2 (Free) mg/l	*BOD mg/l	*Dissolved Iron mg/l	*Dissolved Manganese mg/l	*Dissolved Arsenic mg/l	*Dissolved Cadmium mg/l
Olive WW	18129	22.9	2.68	3.49	45.00	2.00	1938.78	0.24	0.01	0.01	0.00
Brew WW	18130	22.3	0.48	663.77	16.00	14.40	13741.50	0.75	2.55	0.04	0.00
Winery WW	18131	22.6	0.91	6.17	3.40	1.09	2071.43	1.04	0.04	0.02	0.00
AD WW	18132	22.9	6.85	219.66	406.00	14.50	22414.97	1.90	2.00	0.02	0.00

Origin	Lab. Nr.	*Dissolved Chromium mg/l	*Dissolved Copper mg/l	*Dissolved Cyanide mg/l	*Dissolved Lead mg/l	*Dissolved Selenium mg/l	*Dissolved Zinc mg/l	Date Analysed
Olive WW	18129	0.00	0.01	0.20	0.00	0.06	0.06	29/08/2018
Brew WW	18130	0.00	0.06	0.44	0.01	0.02	3.07	29/08/2018
Winery WW	18131	0.00	0.01	0.07	0.00	0.06	0.08	29/08/2018
AD WW	18132	0.00	0.06	0.48	0.00	0.04	0.20	29/08/2018

** = Not SANAS Accredited*

14. APPENDIX F – SELECTED PHYSICAL PROPERTIES OF PVA

Table 13: Physical properties of PVA.

Property	Value	Remarks
Appearance	White granular powder	
Specific gravity	1.19 – 1.31	Increases with degree of crystallinity
Thermal conductivity, W/(m·K)	0.2	
Glass transition temperature, °C	85	99 % hydrolysed
Melting point, °C	200	99 % hydrolysed
Boiling point, °C	228	99 % hydrolysed
Flash point, °C	79.5	
Flammability	Burns (similarly to paper)	
Stability in sunlight	Excellent	
Storage stability	Indefinite (when protected from moisture)	

15. APPENDIX G – SAMPLE CALCULATIONS

Preparation of glycerol (5M):

$$[glycerol] = \frac{(mass\ glycerol)(1\ mol)(1000\ ml)}{(volume\ distilled\ water)(92.09\ g)(1\ L)}$$

$$[glycerol] = \frac{(100\ g)(1\ mol)(1000\ ml)}{(217.19\ ml)(92.09\ g)(1\ L)}$$

$$[glycerol] = 5M$$

COD calculation for wastewater diluted with media (50 % wastewater and 50 % media):

$$COD_{measured} = \varphi_{media}(COD_{media}) + \varphi_{wastewater}(COD_{wastewater})$$

$$1815\ mg/L = 0.5\left(640\frac{mg}{L}\right) + 0.5(COD_{wastewater})$$

$$COD_{wastewater} = 2990\ mg/L$$

Biomass concentration increase:

$$Biomass\ concentration\ increase = \frac{(Cell\ dry\ weight_{\ max} - Cell\ dry\ weight_{initial})}{Cell\ dry\ weight_{max}} \times 100\ \%$$

$$Biomass\ concentration\ increase = \frac{(0.31317\frac{g}{L} - 0.08736\frac{g}{L})}{0.31317\frac{g}{L}} \times 100\ \%$$

$$Biomass\ concentration\ increase = 72.10\ \%$$

COD decrease:

$$COD\ decrease = \frac{(COD_{\ final} - COD_{initial})}{COD_{final}} \times 100\ \%$$

$$COD\ decrease = \frac{(3035\frac{mg}{L} - 1240 mg/L)}{3035\ mg/L} \times 100\ \%$$

$$COD\ decrease = 59.14\ \%$$

Decolourization efficiency:

$$F = \frac{(A_i - A_f)}{A_i} \times 100\ \%$$

$$F = \frac{(0.632 - 0.302)}{0.632} \times 100\ \%$$

$$F = 52.22\ \%$$

Gas composition:

$$Hydrohen\ \% = \frac{Hydrogen\ \%}{Hydrogen\ \% + Carbon\ dioxide\ \%} \times 100\ \%$$

$$Hydrohen\ \% = \frac{18.176}{18.176 + 1.1413} \times 100\ \%$$

$$Hydrohen\ \% = 94.09\ \%$$